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(71) Applicant (for all designated States except US): **ACORDA
THERAPEUTICS, INC.** [US/US]; 15 Skyline Drive,
Hawthorne, NY 10531 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **CAGGIANO, An-
thony, O.** [US/US]; 10 Wildwood Road, Larchmont, NY

10538 (US). **GRUSKIN, Elliott, A.** [US/US]; 23 Beech
Tree Ridge, Killingworth, CT 06419 (US). **SHEPTOVIT-
SKY, Yelena, G.** [US/US]; 62 Mill Brook Road, Stamford,
CT 06942 (US). **KASPERBAUER, Sarah** [US/US]; 3848
Old Crompond Road, Apartment 2C, Cortlandt Manor,
10567 (US).

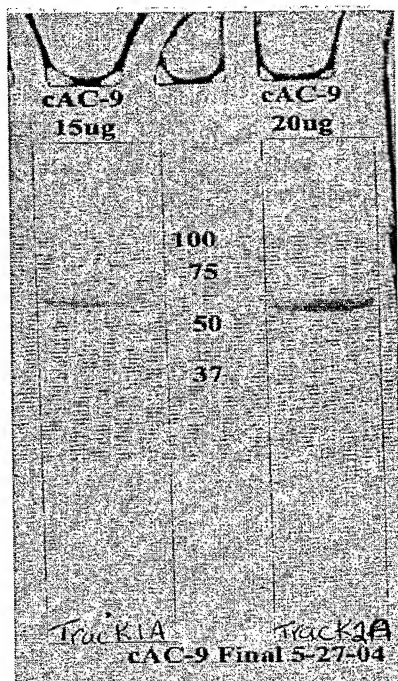
(74) Agent: **MILLER, Raymond, A.**; Pepper Hamilton LLP,
One Mellon Center, 50th Floor, 500 Grant Street, Pitts-
burgh, PA 15219 (US).

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[Continued on next page]

(54) Title: METHODS OF PURIFYING CHONDROITINASE AND STABLE FORMULATIONS THEREOF

Purity yield: 100%



(57) Abstract: An aspect of the present invention relates to stable formu-
lations of chondroitinase and to methods of purifying chondroitinase. The
methods of purifying chondroitinase includes the steps of extracting the en-
zyme from a cell, separating the chondroitinase from the crude cell extract
using cation-exchange chromatography, removing impurities through gel fil-
tration chromatography, and removing endotoxin through an anion-exchange
membrane to produce a purified chondroitinase.

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METHODS OF PURIFYING CHONDROITINASE AND STABLE FORMULATIONS THEREOF

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority from U.S. Provisional Application No. 60/572,030 filed May 18, 2004 entitled "Process of Purification of Chondroitinase," and from U.S. Provisional Application No. 60/621,882 entitled "cABCI Characterization and Formulation" filed October 25, 2004, the contents of which are incorporated herein by reference in their entireties.

FIELD OF THE INVENTION

[0002] One aspect of the present invention relates to a stable formulation of chondroitinase. Another aspect of the present invention relates to methods of purifying chondroitinase.

BACKGROUND

[0003] Proteoglycans, major constituents of the extracellular matrix, are known to be present in large amounts in glial scar tissue and to inhibit recovery following spinal cord injuries (Fawcett & Asher, 1999). Enzymes that are capable of digesting glial scar tissue are an important target for the development of spinal cord injury (SCI) therapeutics. Chondroitinase ABCI (EC 4.2.2.4; cABCI) is a bacterial enzyme that catalyzes the digestion of sulfated chondroitin and dermatan side chains of proteoglycans. This enzyme has been shown to promote functional recovery after spinal cord injury (Bradbury et al., 2002; Caggiano et al., 2005).

[0004] The spinal cord is made up of nerve fibers. Damage to the central nervous system, including the spinal cord, results in a loss of function. Depending upon the type of injury to the central nervous system, the loss of function may manifest itself in loss of sensory, motor or autonomic function or a combination thereof. Sensory functions include

the ability to feel sensations, like pain. Motor functions include the ability to voluntarily move your body. Autonomic functions include involuntary body functions, for example the ability to sweat and breathe.

[0005] The most common types of spinal cord injuries (SCI) include contusions (bruising of the spinal cord) and compression injuries (caused by prolonged pressure on the spinal cord). In contusion injuries, a cavity or hole often forms in the center of the spinal cord. Unlike nerve cells, or neurons of the peripheral nervous system (PNS), neurons of the central nervous system (CNS) do not regenerate after injury.

[0006] Spinal cord injury can be characterized by contusion of the neural tissue with a resultant decrease or loss of the ability of nerve tissue to properly transmit nerve impulses. The usual cause is due to an impact injury of some nature, but it may also occur during the manipulation of the spinal cord in certain surgical procedures. After a spinal cord injury in the adult mammal, the inability of axons to regenerate may lead to loss of sensation, loss of motor function and/or loss of autonomic function, as well as permanent paralysis. One reason that neurons fail to regenerate is their inability to traverse the glial scar that develops following a spinal cord injury. The injury-induced lesion will develop glial scarring, which contains extracellular matrix molecules including chondroitin sulfate proteoglycans (CSPGs). CSPG inhibit nerve tissue growth *in vitro* and nerve tissue regeneration at CSPGs rich regions *in vivo*.

[0007] A number of molecules, and specified regions thereof, have been implicated in the ability to support the sprouting of neurites from a neuronal cell, a process also referred to as neurite outgrowth. The term neurite refers to both axon and dendrite structures. The process of sprouting neurites is essential in neural development and regeneration, especially after physical injury or disease has damaged neuronal cells. Neurites elongate profusely during development both in the central and peripheral nervous systems of all animal species. This phenomenon pertains to both axons and dendrites.

[0008] Various polypeptides, especially cell adhesion molecules (CAMs), have been known to promote neural cell growth. While early efforts in this area of research concentrated on the adhesion-promoting extracellular matrix protein fibronectin (FN), other polypeptides have also been found to promote neural growth. For example, U.S. Patent No. 5,792,743 discloses novel polypeptides and methods for promoting neural growth in the CNS of a mammal by administering a soluble neural CAM, a fragment thereof, or a Fc-fusion product thereof. U.S. Patent No. 6,313,265 discloses synthetic polypeptides containing the pharmacologically active regions of CAMs that can be used in promoting nerve regeneration and repair in both peripheral nerve injuries as well as lesions in the CNS. While helpful, the use of regenerative proteins alone may not be sufficient to effect repair of a damaged nervous system.

[0009] During approximately the past two decades, knowledge of cell adhesion and migration in extracellular matrices (ECMs) at the molecular level has expanded rapidly. The action of enzymes and other polypeptides which degrade components of the extracellular matrix and basement membranes may facilitate the events of neural repair by a variety of mechanisms, including the release of bound cytokines and by increasing the permeability of the matrix, thereby enhancing the mobility of mediator molecules, growth factors and chemotactic agents, as well as the cells involved in the healing process. For example, U.S. Patent No. 5,997,863 discloses the use of glycosaminoglycans to manipulate cell proliferation and promote wound healing.

[0010] Components of the inhibitory CSPGs have been identified as the glycosaminoglycans, chondroitin sulfate (CS) and dermatan sulfate (DS). Removal of these inhibitory molecules would allow neurites to regenerate and reinnervate an area after physical injury or disease, as well as to allow for the recovery of sensory, motor and autonomic functions.

[0011] Previous studies have found that chondroitinases can lyse and degrade CSPGs including, CS and DS. One study found that chondroitinase ABC removed glycosaminoglycan (GAG) chains in and around lesioned areas of rat CNS *in vivo*. The degradation of GAGs promoted expression of a growth-associated protein, GAP-43, indicating an increase in the ability of treated cells to regenerate. However, this growth-associated protein is associated with regeneration in peripheral, but not central, nerve injuries.

[0012] Chondroitin sulfates (CS) are sulfated polysaccharides in linear chains of a repeated disaccharides. They range in molecular weight from about 10,000 to over 100,000 Da. Chondroitin sulfate substrates exist in different isomers designated by the appended letters A, B, and C (Hoffman et al., 1958). The repeating units are composed of uronic acid (GlcA or IdoA) and galactosamine, and are called galactosaminoglycans, and are one example of the glycosaminoglycans, typically abbreviated as GAG. Although these GAG chain species have different repeating disaccharide regions, they are covalently bound through the so-called linkage region tetrasaccharide sequence (see below) to the serine residue in the GAG attachment consensus sequence (Glu/Asp-X-Ser-Gly) of respective core proteins. Chondroitin A and C sulfates (ChS-A, ChS-C) are the most abundant GAGs and are found in cartilage, bone and heart valves. Chondroitin B (ChS-B, or, alternatively, dermatan sulfate) is expressed mostly in skin, blood vessels, heart valves.

[0013] When chondroitinase bacterial preparations were characterized against different chondroitin sulfate (ChS) substrates, a series of distinct chondroitinases were discovered: chondroitinase AC that degrades mostly chondroitin A (ChA) and chondroitin C (ChC) (Yamagata et al., 1968), chondroitinase B that degrades chondroitin (ChB) (Michelacci and Deitrich, 1976), chondroitinase C that acts mostly on ChC (Michelacci & Dietrich CP, 1976) and chondroitinase ABC exhibits specificity against all three substrates - ChS-A, ChS-B and ChS-C (Yamagata et al., 1968, Michelacci et al., 1987).

SUMMARY OF THE INVENTION

[0014] One aspect of the present invention provides stable formulations comprising chondroitinase and a buffer, preferably a sodium phosphate buffer. In one embodiment, a formulation comprising chondroitinase ABCI and about 100 mM sodium phosphate is provided.

[0015] Another aspect of the present invention provides methods of purifying chondroitinase. In one embodiment, the method of purifying chondroitinase comprises extracting the chondroitinase from cells, separating the chondroitinase from the extract, preferably using cation exchange chromatography, removing contaminants and impurities, preferably using gel filtration chromatography, and removing endotoxin, preferably using anion-exchange. The method may further comprise dialysis. The method may also further comprise drying. In a preferred embodiment, the chondroitinase is chondroitinase ABCI. In another preferred embodiment, the chondroitinase is chondroitinase AC.

[0016] Generally, the cells are suspended in a buffer solution containing a surface active agent and sonicated. The chondroitinase is then captured or separated from the extract mixture, preferably by passing the extract through a cation exchange column. Contaminants and impurities are removed from the captured chondroitinase, preferably by gel filtration. Endotoxins are removed from the chondroitinase sample, preferably by an anion exchange column. In one embodiment, the chondroitinase sample may be dialyzed, preferably using a volatile buffer. The chondroitinase may be further processed by drying or lyophilization. In an embodiment, the chondroitinase is chondroitinase ABC. In further embodiment the chondroitinase is chondroitinase AC. In a further embodiment the chondroitinase is a recombinant chondroitinase.

[0017] Another aspect of the invention provides a recombinant expression vector comprising a chondroitinase ABCI with a sequence of SEQ ID NO: 1. Another embodiment

provides a recombinant expression vector comprising a chondroitinase ABCI with a sequence of SEQ ID NO: 2.

DESCRIPTION OF THE DRAWINGS

[0018] In part, other aspects, features, benefits and advantages of the embodiments of the present invention will be apparent with regard to the following description, appended claims and accompanying drawings where:

[0019] Fig. 1. Linear range for product formation rates in cABCI activity assay.

[0020] Fig 2A. Purification of chondroitinase ABCI A280 profile of S200 column. Fig. Fig. 2 B & C: SDS-PAGE of chondroitinase ABCI elution fractions. B – Silver stained. C – Coomassie Blue stained

[0021] Fig. 3. Michaelis-Menten curves for cABCI and substrates.

[0022] Fig. 4A-4E. The SEC light-scattering profiles of the cABCI non-treated (control) and stress-treated. Fig. 4A Freezing/Thawing: Red - control; Blue - 1 cycle; Green - 2 cycles; Purple - 3 cycles; Fig. 4B Exposure to H₂O₂: Red - control; Blue - 0.5mM; Green - 5mM; Purple - 20mM; Fig. 4C Continuous vortexing: Red - control; Blue - 5min; Green - 20min; Purple - 60min; Fig. 4D UV exposure: Red - control; Blue - 40min; Green - 1hr; Purple - 2hr; Fig. 4E Thermal (37°C) stress: Red - control; Blue - 1hr; Green - 4hr; Purple - 20hr.

[0023] Fig. 5. Weak cation-exchange HPLC analysis of the UV-treated cABCI samples. 5A. Initial sample; 5B. after 30 seconds UV exposure; 5C. after 2 minutes UV exposure; 5D. after 5 minutes UV exposure.

[0024] Fig. 6. Comparison of HPLC peak area of ABCI with unit activity.

[0025] Fig. 7. ABCI stability-hand held UV.

[0026] Fig. 8. ABCI stability- exposure to UV in hood.

[0027] Fig. 9. Correlation of cABCI activity data with HPLC profile of cABCI during thermal inactivation. 9A. Histogram of the percent of initial peak areas on HPLC and the percent of initial activity by spectrophotometric assay. 9B. Regression of peak areas and activities by spectrophotometry.

[0028] Fig.10. Dependence of cABCI thermal stability on ionic strength of in the presence of 50 mM Na Phosphate buffer.

[0029] Fig. 11. Dependence of cABCI thermal stability on concentration of buffer species in the presence of 100 mM NaCl and 50 mM NaAcetate..

[0030] Fig. 12. SDS-PAGE analysis of the final purified chondroitinase AC.

DETAILED DESCRIPTION

[0031] Before the present compositions and methods are described, it is to be understood that this invention is not limited to the particular molecules, compositions, methodologies or protocols described, as these may vary. It is also to be understood that the terminology used in the description is for the purpose of describing the particular versions or embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims.

[0032] It must also be noted that as used herein and in the appended claims, the singular forms “a”, “an”, and “the” include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to a “cell” is a reference to one or more cells and equivalents thereof known to those skilled in the art, and so forth. Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of the present invention, the preferred methods, devices, and materials are now described. All publications mentioned herein are incorporated by reference. Nothing herein is to be

construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

[0033] As used herein, the term "about" means plus or minus 10% of the numerical value of the number with which it is being used. Therefore, about 50% means in the range of 45%-55%.

[0034] The term "recombinant protein" refers to a polypeptide of the present invention which is produced by recombinant DNA techniques, wherein generally, DNA encoding a polypeptide is inserted into a suitable expression vector which is in turn used to transform a host cell to produce the protein. Moreover, the phrase "derived from", with respect to a recombinant gene, is meant to include within the meaning of "recombinant protein" those proteins having an amino acid sequence of a native protein, or an amino acid sequence similar thereto which is generated by mutations including substitutions and deletions (including truncation) of a naturally occurring form of the protein.

[0035] The terms "therapeutically effective amount" or "effective amount", as used herein, may be used interchangeably and refer to an amount of a therapeutic compound component of the present invention. For example, a therapeutically effective amount of a therapeutic compound is a predetermined amount calculated to achieve the desired effect, i.e., to effectively treat an injury to the central nervous system. For example, a therapeutic compound comprising a therapeutically effective amount of chondroitinase which may be purified by a method of the present invention and formulated to provide a stable, active enzyme, is sufficient to degrade the CSPGs of the lesioned area of the spinal cord or an amount sufficient to restore, in whole or in part, motor, sensory or autonomic function of the mammal and may result in a regeneration of neurons in a central nervous system, such as by promoting axonal growth into an injured area.

[0036] The term "vector" refers to a vehicle which can transport the nucleic acid molecules. The nucleic acid molecules encoding the chondroitinase polypeptide are

covalently linked to the vector nucleic acid. With this aspect of the invention, the vector can be a plasmid, single or double stranded phage, a single or double stranded RNA or DNA viral vector, or artificial chromosome, such as a BAC, PAC, YAC, OR MAC.

[0037] Chondroitinase as used herein include, but are not limited to, chondroitinase ABCI, chondroitinase ABCII, chondroitinase AC, chondroitinase B or mammalian enzymes with chondroitinase-like activity such as Hyal1, Hyal2, Hyal3, Hyal4 and PH2O.

[0038] Chondroitinase may be obtained from a microorganism that naturally expresses a chondroitinase; for example, but not limited to, *E. coli*, *Proteus vulgaris* or from the expression of a recombinant protein in a host cell. The host cell can be a prokaryotic cell (such as *E. coli*) or a eukaryotic cell (such as yeast, a mammalian cell or an insect cell).

[0039] In one embodiment of the invention, a recombinant chondroitinase ABCI from *Proteus vulgaris* was over-expressed in *E.coli*. The primary sequence of this protein is shown below:

ATSNPAFDPKNLMQSEIYHFAQNNPLADFSSDKNSILTLSDKRSIMGNQSLWKKWG
GSSFTLHKKLIVPTDKEASKAWGRSSTPVFSFWLYNEKPIDGYLTIDFGEKLISTSEAQ
AGFKVKLDFTGWRTVGVSLNNDLENREMTLNATNTSSDGTQDSIGRSLGAKVDSIRF
 KAPSNVSQGEIYIDRIMFSVDDARYQWSDYQVKTRLSEPEIQFHNVKPQLPVT PENLA
 AIDLIRQRLINEFVGGEKETNLALEENISKLSDFDALNTHTLANGGTQGRHLITDKQI
 IYQPENLNSQDKQLFDNYVILGNYTTLMFNISRAYVLEKDPTQKAQLKQMYLLMTK
 HLLDQGFVKGSALVTTHHWGYSSRWYISTLLMSDALKEANLQTQVYDSSLWYSR
 EFKSSFDMKVSADSSDLDFNTLSRQHLALLLEPDDQKRINLVNTFSHYITGALTQV
 PPGKGDGLRPDGTAWRHEGNYPGYSFPAFKNASQLIYLLRDTPFVSGESGWNLSKK
 AMVSAWIYSNPEVGLPLAGRHLNPSLSKVAQGYWLAMS AKSSPDKTLASIYLAI
 SDKTQNESTAIFGETITPASLPQGFYAFNGGAFGIHRWQDKMVT LKAYNTNVWSSEI
 YNKDNRYGRYQSHGVAQIVSNGSLSQGYQQEGWDWNRMPGATTIHLPLKDLDSP
 KPHTLMQRGERGFSGTSSLEGQYGMMAFDLIYPANLERFDPNFTAKKSVLAADNHLI
 FIGSNINSSDKNKNVETTLFQHAIPTLNTLWINGQKIENMPYQTTLQQGDWLIDSNG
 NGYLITQAEKVNVSRQHQVSAENKNRQPTTEGNFSSAWIDHSTRPKDASYEYMVFLD
 ATPEKMGEMAQKFRENGLYQVLRKDKDVHILDKLSNVTGYAFYQPASIEDKWIK
 KVNKPAIVMTHRQKDTLIVSAVTPDLNMTRQKAATPVTINVTINGKWQSADKNSEV
 KYQVSGDNTELTFTSYFGIPQEIKLSPLP (SEQ ID NO:1),

wherein bolded and underlined residues indicate residues that do not correlate with those

within the GeneBank sequences and italicized residues indicate a peptide sequence that was reported to be cleaved from the processed enzyme (Khandke, 1996).

[0040] In another embodiment, a recombinant chondroitinase can be produced from the amino acid sequence of the processed enzyme having the sequence:

QDSIGRSLGAKVDSIRFKAPSNVSQGEIYIDRIMFSVDDARYQWSDYQVKTRLSEPEI
QFHNVPKQLPVTPENLAIDLIRQRLINEFVGGEKETNLALEENISKLSKSDFDALNTHT
LANGGTQGRHLITDKQIIYQPENLNSQDKQLFDNYVILGNYTTLMFNISRAYVLEKD
PTQKAQLKQMYLLMTKHLLDQGFVKGSALVTTHHWGYSSRWYISTLLMSDALKE
ANLQTQVYDSLLWYSREFKSSFDMKVSADSSDLDFNTLSRQHLALLLLEPDDQKRI
NLVNTFSHYITGALTQVPPGGKDGLRPDGTAWRHEGNYPGYSFPAFKNASQLIYLLR
DTPFSVGESGWNSLKKAMVSAWIYSNPEVGLPLAGRHPNPSPLKSVAQGYWLA
MSAKSSPDKTLASIYLAISDKTQNESTAIFGETITPASLPQGFYAFNGGAFGIHRWQDK
MVTLKAYNTNVWSSEIYNKDNRYGRYQSHGVAQIVSNGSQLSQGYQQEGWDWNR
MPGATTIHLPLKDLDSPKPHITLMQRGERGFSGTSSLEGQYGMMAFDLIYPANLERFD
PNFTAKKSVLAADNHLIFIGSNINSSDKNKNVETTLFQHAIPTLNTLWINGQKIENMP
YQTTLQQGDWLIDSNNGYLYITQAEKVNVSQRHQVSAENKNRQPTEGNFSSAWIDH
STRPKDASYEYMFVLDATPEKMGEMAQKFRENGLYQVLRKDKDVHILDKLSNVT
GYAFYQPASIEDKWIKKVNKPAIVMTHRQKDTLIVSAVTPDLNMTRQKAATPVTINV
TINGKWQSAADKNSEVKYQVSGDNTELTFTSYFGIPQEIKLSPLP (SEQ ID NO:2).

[0041] Expression of a recombinant chondroitinase gene can be produced by ligating a nucleic acid encoding a chondroitinase protein, or a portion thereof, into a vector suitable for expression in either prokaryotic cells, eukaryotic cells, or both. Procedures for ligation are well known to those of ordinary skill in the art. Expression vectors for production of recombinant forms of the subject chondroitinase polypeptides include plasmids and other vectors. For instance, suitable vectors for the expression of a chondroitinase polypeptide include plasmids of the types: pBR322-derived plasmids, pEMBL-derived plasmids, pEX-derived plasmids, pBTac-derived plasmids and pUC-derived plasmids for expression in prokaryotic cells, such as *E. coli*.

[0042] A number of vectors exist for the expression of recombinant proteins in yeast. For instance, YEP24, YIP5, YEP51, YEP52, pYES2, and YRP17 are cloning and expression vehicles useful in the introduction of genetic constructs into *S. cerevisiae* (see, for example, Broach et al. (1983) in *Experimental Manipulation of Gene Expression*, ed. M. Inouye Academic Press, p. 83, incorporated by reference herein). These vectors can replicate in *E. coli* due to the presence of the pBR322 origin of replication, and in *S. cerevisiae* due to the

replication determinant of the yeast 2 micron plasmid. In addition, drug resistance markers such as ampicillin may be used.

[0043] In another embodiment, a chondroitinase polypeptide is produced recombinantly utilizing an expression vector generated by subcloning the coding sequence of one of the chondroitinase proteins represented in SEQ ID NO: 1 or SEQ ID NO: 2.

[0044] Mammalian expression vectors may contain prokaryotic sequences, to facilitate the propagation of the vector in bacteria, and one or more eukaryotic transcription units that are expressed in eukaryotic cells. The pcDNAI/amp, pcDNAI/neo, pRc/CMV, pSV2gpt, pSV2neo, pSV2-dhfr, pTk2, pRSVneo, pMSG, pSVT7, pko-neo and pHyg derived vectors are examples of mammalian expression vectors suitable for transfection of eukaryotic cells. Some of these vectors are modified with sequences from bacterial plasmids, such as pBR322, to facilitate replication and drug resistance selection in both prokaryotic and eukaryotic cells. Alternatively, derivatives of viruses such as the bovine papillomavirus (BPV-1), or Epstein-Barr virus (pHEBo, pREP-derived and p205) can be used for transient expression of proteins in eukaryotic cells. The various methods employed in the preparation of the plasmids and transformation of host organisms are well known in the art. For other suitable expression systems for both prokaryotic and eukaryotic cells, as well as general recombinant procedures, see *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989) Chapters 16 and 17, there references are incorporated herein.

[0045] In some instances, it may be desirable to express the recombinant chondroitinase polypeptide by the use of an insect expression system such as the baculovirus expression system. Examples of such baculovirus expression systems include pVL-derived vectors (such as pVL1392, pVL1393 and pVL941), pAcUW-derived vectors (such as pAcUW1), and pBlueBac-derived vectors (such as the β -gal containing pBlueBac III).

[0046] The expression vectors listed herein are provided by way of example only and represent the well-known vectors available to those of ordinary skill in the art that may be useful to express the nucleic acid molecules. The person of ordinary skill in the art would be aware of other vectors suitable for maintenance propagation or expression of the nucleic acid molecules described herein. These are found for example in Sambrook, J., Fritsh, E. F., and Maniatis, T. *Molecular Cloning: A Laboratory Manual*. 2nd, ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989, the text of which is incorporated herein.

[0047] When it is desirable to express only a portion of a chondroitinase protein, such as a form lacking a portion of the N-terminus, i.e. a truncation mutant which lacks the signal peptide, it may be necessary to add a start codon (ATG, which encodes the amino acid methionine) to the oligonucleotide fragment containing the desired sequence to be expressed. It is well known in the art that a methionine at the N-terminal position can be enzymatically cleaved by the use of the enzyme methionine aminopeptidase (MAP). MAP has been cloned from *E. coli* (Ben-Bassat et al. (1987) *J. Bacteriol.* 169:751-757) and *Salmonella typhimurium* and its in vitro activity has been demonstrated on recombinant proteins (Miller et al. (1987) *PNAS* 84:2718-1722). Therefore, removal of an N-terminal methionine, if desired, can be achieved either in vivo by expressing chondroitinase-derived polypeptides in a host which produces MAP (e.g., *E. coli* or CM89 or *S. cerevisiae*), or in vitro by use of purified MAP.

[0048] Expression vectors contain cis-acting regulatory regions that are operably linked in the vector to the chondroitinase nucleic acid such that transcription of the nucleic acid molecules is allowed in a host cell.

[0049] The recombinant host cells are prepared by introducing the vector constructs into the cells by techniques readily available to the person of ordinary skill in the art. These include, but are not limited to, calcium phosphate transfection, DEAE-dextran-mediated

transfection, cationic lipid-mediated transfection, electroporation, transduction, infection, lipofection, and other techniques such as those found in Sambrook, et al. (*Molecular Cloning: A Laboratory Manual. 2nd, ed.*, Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989).

[0050] As is known in the art, chondroitinase polypeptides can be produced by standard biological techniques or by chemical synthesis. For example, a host cell transfected with a nucleic acid vector directing expression of a nucleotide sequence encoding the subject polypeptides can be cultured under appropriate conditions to allow expression of the peptide to occur. The chondroitinase polypeptide may be secreted and isolated from a mixture of cells and medium containing the recombinant chondroitinase polypeptide. Aspects of the invention described herein provide purification methods wherein the chondroitinase is isolated in a pure form that is more stable and active than those methods currently used.

[0051] Alternatively, the peptide may be retained cytoplasmically by removing the signal peptide sequence from the recombinant chondroitinase gene and the cells harvested, lysed and the protein isolated by the purification methods described herein.

[0052] According to one aspect of the invention, the process of purifying chondroitinase comprises the following steps: 1) extracting the enzyme from a cell, 2) separating the crude cell extract using cation-exchange chromatography, 3) further separating the extract by a gel filtration chromatography, and 4) removing endotoxin through an anion-exchange membrane to produce a purified chondroitinase, which exhibits high activity relative to chondroitinase purified by conventional methods.

[0053] Extraction of chondroitinase from cells can be more effective by using a buffer solution to which a surfactant is added. A surfactant is a surface active agent that has solubilizing tendencies and that contains groups of opposite polarity. These agents may be used to disrupt the integrity of a cell. Thus, a surfactant may be used to extract an enzyme

from a cell. Any surfactant which can promote the extraction of chondroitinase from a cell can be used in the present invention, preferably the surfactant is a nonionic surfactant.

[0054] Nonionic surfactants which can be used include, but are not limited to, polyoxyethylene alkyl ethers, polyoxyethylene p-t-octylphenyl ethers, polysorbate, and the like. Emulgen-type surfactants, Liponox-type surfactants, Brij-type surfactants, and the like are given as specific examples of polyoxyethylene alkyl ethers. Commercially available surfactants among these are Emulgen 120, Emulgen 109P, Liponox DCH, Brij 35, 78, 76, 96, 56, 58, 98, Nikkol BL-9EX, BL-21, BL-25, and the like. Given as specific examples of polyoxyethylene p-t-octylphenyl ethers are Triton-type surfactants, Nonidet P40-type surfactants, Igepal/CA-type surfactants, Polytergent G, Neutronyx-type surfactants, Conco-type surfactants, and the like. Among these types of surfactants, Triton X-100, X-45, X-114, X-102, X-165, X-305, X-405, Nonidet P-40, Igepal CA-630, Neutronyx 605, Conco NIX-100, and the like are commercially available. Tween-type surfactants, Emasol-type surfactants, Sorbester-type surfactants, Crill-type surfactants, and the like are given as specific examples of polysorbates. Sorbitan mono-9-octadecanoate poly(oxy-1,2-ethanediyl) derivatives, commercially available as Tween 80 are preferred as polysorbate and the like.

[0055] Of the above surfactants, Triton X surfactants are preferred, including but not limited to Triton X-114. Generally, the detergent such as, but not limited to, Triton X, can be added to the sample of cells to be extracted. In one embodiment of the invention, the concentration of the detergent can range from about 0.1% to about 10%(v/v), preferably in the range of about 0.1% to about 3%(v/v), or more preferably in the range of about 0.2% to about 2%.

[0056] In one embodiment of the invention, the process of extraction may also involve sonication. Sonication involves the use of sound waves to disrupt fragile cells (made fragile, for example, by exposure to a surfactant, such as Triton X). This results in dispersing and disrupting the cells such that the integrity of the cell is further destroyed, thereby causing

the release of intracellular components. Sonication may involve either or both a pulsed or continuous exposure to sound waves. For example, to sonicate the cells, a small (micro) probe can be used. The sonicator can be set to pulse (not continuous). In another embodiment, the sonicator can be set to continuous. In a further embodiment, the sonication step may use a combination of pulsed and continuous sonication. In one embodiment, the cell suspension may be sonicated with 10 short burst of 10 seconds followed by intervals of 30 seconds for cooling. The cell suspension may be kept on ice during sonication to avoid overheating of the sample constituents. Following sonication the cell debris may be removed by centrifugation. Other methods of sonication may be employed, as readily determined by those skilled in the art, depending on the cell type being disrupted.

[0057] In another embodiment, enzyme extraction may also involve polytron homogenization. This process will mechanically grind up the surfactant treated cells, therein disrupting cell integrity, and releasing cell components into a solution for further purification. Generally, the sample is maintained on ice to prevent or limit any heating of the sample. The sample may be homogenized for about 30 seconds, or until the cell clumps have been dispersed. Methods for performing polytron homogenization are well known in the art.

[0058] The chondroitinase may be captured from the cell extract using ion exchange chromatography. In ion exchange chromatography, charged substances are separated using column materials that carry an opposite charge. Two exchanger types are differentiated: basic (positively charged) and acidic (negatively charged). The ion exchanger types can be further divided into weakly basic or acidic or strongly basic or acidic. With strongly basic or acidic materials all functional groups are generally present in the ionized form. For example, the quaternary amino groups (R_3N^+) are positively charged, while the sulfonic acid groups (SO_3^-) are negatively charged. The weakly basic types and weakly acidic types of ion exchange columns also exist. The weakly basic types are generally secondary and tertiary amino functional groups; the weakly acidic types are generally carboxyl functional groups.

[0059] Many proteins can be separated as polyanions ($\text{pH} > \text{pI}$) or as polycations ($\text{pH} < \text{pI}$). The most common ion exchanger groups include, but are not limited to, imethylammoniummethyl (anion), diethylaminoethyl (anion), dimethylaminoethyl (anion), carboxy (cation), carboxyalkyl (cation), sufoisobutyl (cation), sulphoalkyl (cation), sulphopropyl (cation) and sulfoethyl (cation).

[0060] To capture the enzyme from the cell extract, the extract obtained may be subjected to cation exchange chromatography. Using a cation exchange resin produces a chondroitinase with increased activity and purity compared to the crude lysate. Weak or strong cation exchange resins may be used, for example, but not limited to, cation exchange resins having a carboxyalkyl group and a sulfoalkyl or sulphopropyl group, respectively. Other cation exchange resins are well known in the art (see above).

[0061] Thus, in one embodiment of the invention the enzyme may be captured from the cell extract by loading the sample onto the cation exchange chromatography, washing of the cation exchanger, in which the cell components other than chondroitinase are washed out by increasing the ion strength and/or by pH changes, i.e. under cation-exchange chromatography conditions; and elution of the chondroitinase sample by a further increase in the ion strength and/or by a pH change.

[0062] Buffers used in cation exchange chromatography include, but are not limited to those listed in Table 1.

Table 1. Cation Exchange Chromatography Buffers

Molecule	pKa	dpKa/degree C.	Counter ion
Maleic acid	2.00		sodium
Malonic acid	2.88		sodium
citric acid	3.13	-0.0024	sodium
lactic acid	3.81		sodium
formic acid	3.75	0.0002	sodium or lithium
butaneandioic acid	4.21	-0.0018	sodium
acetic acid	4.76	0.0002	sodium or lithium

malonic acid	5.68		sodium or lithium
phosphate	7.20	-0.0028	sodium
HEPES	7.55	-0.0140	sodium or lithium
BICINE	8.35	-0.0180	sodium

[0063] Removal of aggregates and low molecular weight contaminants and impurities may be carried out through various filtration methods including, for example, gel filtration or size exclusion chromatography. Commercially available examples of gel filtration are Sephadex and Sephacryl.

[0064] Gel filtration chromatography is a separation based on size. It is also called molecular exclusion or gel permeation chromatography. In gel filtration chromatography, the stationary phase consists of porous beads with a well-defined range of pore sizes. The stationary phase for gel filtration is said to have a fractionation range, meaning that molecules within that molecular weight range can be separated.

[0065] Thus, proteins that are small enough can fit inside all the pores in the beads and are said to be included. These small proteins have access to the mobile phase inside the beads as well as the mobile phase between beads and elute last in a gel filtration separation. Proteins that are too large to fit inside any of the pores are said to be excluded. They have access only to the mobile phase between the beads and, therefore, elute first. Proteins of intermediate size are partially included - meaning they can fit inside some but not all of the pores in the beads. These proteins will then elute between the large ("excluded") and small ("totally included") proteins.

[0066] Another contaminant that may be present in the cell lysate preparation is endotoxin. Endotoxin is a common toxic contaminant in biological systems. It is important to sufficiently remove endotoxin which is a component of the cell wall of bacteria. Endotoxin is a lipopolysaccharide in the cell wall of most gram-negative bacteria such as *E.coli*. Endotoxin included in proteins is known to cause symptoms of high fever, endotoxin

shock, and inflammation even in a very small amount. Since bacterial extracts may be highly contaminated with endotoxin, embodiments of the invention may include an endotoxin removal step in a purification process. Various methods may be employed for the removal of endotoxin such as, but not limited to, cation exchange chromatography, anion exchange chromatography, affinity chromatography, ultrafiltration, and phase-separation using a surfactant.

[0067] In one embodiment of the invention, the endotoxin is removed using an anion exchange column. Examples of anion exchange chromatography include, but are not limited to, Q-membrane, a quaternary amine; and diethylaminoethane (DEAE) resin. Buffers used in anion exchange chromatography include, but are not limited to those illustrated in Table 2.

Table 2. Anion Exchange Chromatography Buffers

Molecule	pKa	dpKa/degree C.	Counter ion
N-methyl piperazine	4.75	-0.015	chloride
piperazine	5.68	-0.015	chloride or formate
L-histidine	5.96		chloride
bis-Tris	6.46	-0.017	chloride
bis-Tris propane	6.80		chloride
triethanolamine	7.76	-0.020	chloride or acetate
Tris	8.06	-0.028	chloride
N-methyl-diethanolamine	8.52	-0.028	chloride
diethanolamine	8.88	-0.025	chloride
1,3-diaminopropane	8.64	-0.031	chloride
ethanolamine	9.50	-0.029	chloride
piperazine	9.73	-0.026	chloride
1,3-diaminopropane	10.47	-0.026	chloride
piperidine	11.12	-0.031	chloride
phosphate	12.33	-0.026	chloride

[0068] Partitioning may occur at various steps of the process, preferably during purification prior to gel filtration. Filtration through Q membranes is another alternative for endotoxin cleaning step. In one embodiment, Q membrane filtration may be employed using a pH of pH 5.5 in 20 mM NaAcetate and 100 mM NaCl. According to K.C. Hou and R. Zaniewski, *Biotech. Appl. Biochem.* 12, 315-324, 1990, these pH and salt conditions are expected to remove in the range of about 70 to 85% endotoxin. In one embodiment a Q filtration process in a flowthrough collection mode yields greater than about 95% of chondroitinase. Q membrane filtration may be performed at various times during the process, including, for example, at the end of purification after gel filtration.

[0069] Thus, in one embodiment of the invention the removal of the endotoxin from the chondroitinase in the sample may include loading the sample onto the anion exchange chromatography, washing of the anion exchanger, in which the impurities are washed out by increasing the ion strength and/or by pH changes, *i.e.* under anion-exchange chromatography conditions; and elution of the chondroitinase sample by a further increase in the ion strength and/or by a pH change.

[0070] Dialysis is one of the most commonly utilized methods for transferring a biological sample, usually protein based, from one media to another. It is frequently necessary to remove salts or change the buffer after one step in the purification for the next step to work efficiently. This may be achieved by dialysis wherein the protein solution is kept in a semi-permeable membrane and placed in the buffer so that small molecules *e.g.* salts, can pass freely across the membrane whilst large molecules *e.g.* proteins, are retained. One embodiment of the present invention includes a dialysis step to further purify the chondroitinase. A volatile buffer, such as ammonium bicarbonate, pH 8.0 may be used for the dialysis step. Other buffers may also be used. The choice of buffer chosen should be one that is appropriate for the protein being dialyzed. Such buffers are well known in the art (for example, but not limited to, Tris based buffers, phosphate buffers, etc). The buffer used for

dialysis may be any buffer that is able to maintain the appropriate pH in which to stabilize the protein being isolated.

[0071] For storage and distribution of the purified chondroitinase, the process of purifying the chondroitinase may further include the step of drying. The step of drying may involve conventional heat drying or more preferably, lyophilization or freeze drying.

[0072] Embodiments of the invention may include a method of monitoring the enzyme yields and purity profiles by reverse-phase HPLC is further provided. This may be performed following any or all of the steps in the purification process.

[0073] In one embodiment of the invention the final enzyme yield can be up to about 50 mg chondroitinase from 1L cultured cells. In a further embodiment the final enzyme yield can be in the range of about 75 to 85 mg/1 L of cells.

[0074] The purified chondroitinase of the present invention may be characterized by one or more of the following properties: enzyme activity, the pI, the substrate specificity, the rate of substrate catalysis, the inhibitory effect of divalent metal salts, the optimal storage buffer pH, the effects of various stress conditions, the optimal buffer and ionic strength, stabilization of the enzyme in various excipients, and the effect of enzyme concentration on thermal stability.

[0075] A chondroitinase ABCI was used as an example of a chondroitinase that can be purified and formulated according to the embodiments of the invention. The lyophilized purified chondroitinase ABCI was reconstituted and assayed for activity which was compared to activities of chondroitinase ABCI enzymes available from other sources. The activity of the chondroitinase ABCI enzyme of the present invention was a relatively high enzyme preparation. The activity of the purified chondroitinase ABCI is about 160 U/mg. The pI of the purified chondroitinase ABCI of the present invention is about 7.8 to about 8.0. The affinity of the purified chondroitinase ABCI is similar for chondroitin A, chondroitin B, and chondroitin C. The rate of catalysis of a substrate for the purified chondroitinase ABCI of the

present invention is greater for chondroitin A than chondroitin C, which is greater than the rate of catalysis of chondroitin B.

[0076] Divalent metal salts may inhibit an activity of chondroitinase. For example, the purified chondroitinase ABCI may be inhibited by Zn, Ni and Co. Ca and Mg appear to be less inhibitory. Storage buffer pH does not affect the activity of the purified chondroitinase. In preferred embodiments, the storage buffer is pH 7.4, physiological pH.

[0077] While chondroitinase generally may be affected by various adverse conditions, the purified chondroitinase of the present invention does not appear to be affected by repeated freezing and thawing.

[0078] Various embodiments provide a stable formulation of the enzyme for both storage and administration. Generally, the chondroitinase of such stable formulations exhibit at least about 50% of activity at about 24 hours, preferably at least about 75% of activity, more preferably at least about 85% of activity. In another aspect of the invention, the formulations consistently provide stable chondroitinase activity.

[0079] In one embodiment, the chondroitinase is formulated in a phosphate buffer, preferably a sodium phosphate buffer with a concentration in the range of about 50 mM to about 1 M. A preferred embodiment is about 750 mM sodium phosphate. Another preferred embodiment is about 100 mM sodium phosphate. In a further embodiment the chondroitinase may be formulated in a sodium phosphate buffer that further comprises sodium acetate. Sodium acetate may be present in the range of 25 mM to about 75 mM. In a preferred embodiment the sodium acetate concentration is about 50 mM. In one embodiment a preferred formulation for administration is a chondroitinase in a buffer with a pH of about 7.4. Further embodiments of formulations for storage and administration are provided in the Examples described.

[0080] In further embodiment, a formulation comprising purified chondroitinase and a buffer comprising an increased ionic strength is provided. Embodiments wherein a

formulation comprises an increased ionic concentration may increase stability of an enzyme formulation. For example, a preferred embodiment provides a formulation with about 1 M NaCl in sodium phosphate. The concentration of sodium phosphate may be about 50 mM. In a preferred embodiment, the chondroitinase storage concentration is below about 0.4 mg/ml.

[0081] In one embodiment, a chondroitinase ABC formulation comprises about 0.4 mg/ml of chondroitinase ABC in about 100 mM Na phosphate, at a pH of about 7.4 with a preferred substrate specificity for chondroitin A, B, and C about the same. In another embodiment, a formulation comprising a chondroitinase B with a purified chondroitinase ABC is provided.

[0082] In another embodiment, a chondroitinase AC purification is provided comprising the following steps: 1) extracting the enzyme from a cell, 2) separating the crude cell extract using cation-exchange chromatography, 3) further separating the extract by a gel filtration chromatography, and 4) removing endotoxin through an anion-exchange membrane to produce a purified chondroitinase AC. In an embodiment a purified chondroitinase AC is dialyzed into a volatile buffer, lyophilized and stored at -80°C . Example 12 describes one embodiment of a method of purification of a chondroitinase AC.

[0083] In one embodiment, reconstitution and about 4°C storage in a buffer at about 0.1M sodium phosphate, pH 7.4, 50 mM sodium acetate is provided. In another embodiment, stabilizing buffer (for about 37°C studies) at about 0.75M sodium phosphate, pH 7.4, 50 mM sodium acetate is provided. In another embodiment storage of chondroitinase is in the lyophilized form.

[0084] Chondroitinase activity can be stabilized by the addition of excipients or by lyophilization. Stabilizers include carbohydrates, amino acids, fatty acids, and surfactants and are known to those skilled in the art. Examples include carbohydrates such as sucrose, lactose, mannitol, and dextran, proteins such as albumin and protamine, amino acids such as arginine, glycine, and threonine, surfactants such as TWEEN® and PLURONIC®, salts such

as calcium chloride and sodium phosphate, and lipids such as fatty acids, phospholipids, and bile salts.

[0085] The stabilizers are generally added to the protein in a ratio of 1:10 to 4:1, carbohydrate to protein, amino acids to protein, protein stabilizer to protein, and salts to protein; 1:1000 to 1:20, surfactant to protein; and 1:20 to 4:1, lipids to protein. Other stabilizers include high concentrations of ammonium sulfate, sodium acetate or sodium sulfate, based on comparative studies with heparinase activity. The stabilizing agents, preferably the ammonium sulfate or other similar salt, are added to the enzyme in a ratio of 0.1 to 4.0 mg ammonium sulfate/IU enzyme.

[0086] Chondroitinase may be administered topically, locally or systemically. Topical or local administration is preferable for greater control of application. The chondroitinases, singularly or in combination, can be mixed with an appropriate pharmaceutical carrier prior to administration. Examples of generally used pharmaceutical carriers and additives are conventional diluents, binders, lubricants, coloring agents, disintegrating agents, buffer agents, isotonicizing fatty acids, isotonicizing agents, preservatives, anesthetics, surfactants and the like, and are known to those skilled in the art. Specifically pharmaceutical carriers that may be used are dextran, sucrose, lactose, maltose, xylose, trehalose, mannitol, xylitol, sorbitol, inositol, serum albumin, gelatin, creatinine, polyethylene glycol, non-ionic surfactants (e.g. polyoxyethylene sorbitan fatty acid esters, polyoxyethylene hardened castor oil, sucrose fatty acid esters, polyoxyethylene polyoxypropylene glycol) and similar compounds. Pharmaceutical carriers may also be used in combination, such as polyethylene glycol and/or sucrose, or polyoxyethylene sorbitan fatty acid esters, polyoxyethylene sorbitan monooleate (20 E. 0.) is particularly preferred.

[0087] A treatment regimen according to the invention may be carried out by a means of administering, alone or a combination of thereof, chondroitinase ABCI, chondroitinase ABCII, chondroitinase AC and chondroitinase B or mammalian enzymes with

chondroitinase-like activity such as Hyal1, Hyal2, Hyal3, Hyal4 and PH2O to the lesions of the injured area of the CNS. The mode of administration, the timing of administration and the dosage are carried out such that the functional recovery from impairment of the CNS is enhanced by the promotion of neurite outgrowth. The treatments of the present disclosure deliver an effective amount of chondroitinase ABCI purified according to the present invention alone or in combination with chondroitinase ABCII, chondroitinase AC and chondroitinase B or mammalian enzymes with chondroitinase-like activity such as Hyal 1, Hyal 2, Hyal 3, Hyal 4 and PH2O to the injured site.

[0088] The effective amount of chondroitinase can be administered in a single dosage, two dosages or a plurality of dosages. Although it is to be understood that the dosage may be administered at any time, in one embodiment, the dosage is administered within 12 hours after injury, or as soon as is feasible. In another embodiment, the dosage is administered to an injured mammal in one, two or a plurality of dosages; such dosages would be dependant on the severity of the injury and the amount of CSPGs present in the glial scarring. Where a plurality of dosages is administered, they may be delivered on a daily, weekly, or bi-weekly basis. The delivery of the dosages may be by means of catheter or syringe. Alternatively, the treatment can be administered during surgery to allow direct application to the glial scar.

[0089] As an example of a purified formulation of a chondroitinase a recombinant ABCI (cABCI) was purified and characterized using the methods of the present invention using the following parameters: temperature stability, enzyme characteristics, susceptibility to various stress conditions, degradation products; effects of different excipients on enzyme stability.

[0090] The following methods are used to illustrate the various embodiments of the present invention. The methods are exemplary methods and are not meant to limit the invention.

[0091] Activity assay. The enzymatic activity of cABCI was assayed according to a modified version from Hamai et al. (1997). 125 μ l of reaction mixture containing 40mM Tris, pH 8.0. 40mM NaAcetate, 0.002% casein was incubated at about 37°C for at least 3 min. After incubation, 1mg/ml (final concentration) chondroitin C sulfate and 0.05-0.5 μ g cABCI enzyme were added, the mixture was gently vortexed and then the product formation rate was monitored by the absorption at about 232nm for about 45-90 sec. Calculations for substrate and product concentrations were based on MW of hexuronate residues equaling 521 and the molar extinction coefficient (ϵ^{232}) for unsaturated hexuronate-6-sulfate at 232nm of 5,500. When chondroitin A and B are used as substrates in the assay, the calculations for unsaturated hexuronate-4-sulfate products were done with MW equaling 503 and an ϵ^{232} of 5,100. Initial activity rates were calculated in nmole of disaccharide/min by fitting the collected data into a linear function. Specific enzyme activity was expressed in U/mg, where unit (U) is defined as μ mole of product formed within 1 min. The linear range for measuring chondroitin degradation rate was wide as shown in **Figure 1**.

[0092] Estimation of extinction coefficient. The extinction coefficient for different batches of cABCI was determined. Two different batches of purified chondroitinase ABCI were reconstituted in 20 mM sodium acetate, pH 5.5, 100 mM NaCl. Some samples contained 0.3M sucrose in the reconstitution buffer. Absorption at 280nm and protein concentrations using modified Lowry protein assay measurements were taken for each sample. Estimation of extinction coefficient for cABCI is presented in Table 3 below.

Table 3. Extinction Coefficient

cABCI sample	A280	Concentration, mg/ml	0.1% ϵ_{280}
Batch 5c-4°C	0.928	0.68	1.36
Batch 5c-4°C sucrose	0.53	0.4	1.32
Batch 5c-T _{room}	0.672	0.342	1.96
Batch 5c-T _{room} sucrose	0.46	0.25	1.84
Batch 5d	1.81	0.98	1.84
			Average

			1.66
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[0093] Extinction coefficient estimate for 0.1% cABCI solution was derived by dividing A280 by concentration (mg/ml). The averaged extinction coefficient (1.66) was used in further experiments for cABCI concentration measurements.

[0094] Size exclusion chromatography (SEC) characterization. Analytical SEC was used to characterize aggregation and conformation of the chondroitinase ABCI. Analytical SEC was performed using a Shodex KW-803, which has a separation range of about 50,000 to 150,000 Daltons (Da) and Shodex KW-804 columns, which has a separation range of about 100,000 to 600,000 Daltons (Da). The buffer for the mobile phase was 100 mM sodium phosphate, 50 mM NaCl, 0.5% betaine, pH 7.3. The analysis was done at flow rate 1 ml/min at ambient temperatures (approximately 22°C).

[0095] Protein assay. To determine the protein concentration a modified Lowry protein assay (BioRad) and BCA (Pierce) were used according to supplier instructions.

[0096] SDS-PAGE. Proteins were separated on a 4-20% gradient SDS-PAGE precast minigel (BioRad) and electrophoresis was carried out at 200V in a minigel apparatus (BioRad). The gels were then stained with either Coomassie or Silver stain.

[0097] IEF-PAGE. IEF-PAGE was carried out to determine the pI value for chondroitinase ABCI in a 3-10 pH range using NOVEX IEF gels (Invitrogen) and run according to manufacturer's instructions using a NOVEX gel apparatus. The gels were stained with Colloidal Blue Coomassie.

[0098] Western blotting. The proteins were separated on SDS-PAGE and then electroblotted onto nitrocellulose membrane by the tank-transfer method (BioRad) according to the manufacturer's instructions. The transfer buffer contained 25 mM Tris and 192 mM Glycine at pH 8.3, 1% SDS.

[00099] Oxyblot assay. Detection of carbonyl groups that are introduced into protein side chains by a site-specific mechanism was provided by OxyBlot Protein Oxidation Detection kit from Chemicon International. Specifically, the carbonyl groups in the protein side chains were derivatized by 2,4-dinitrophenylhydrazine (DNPH) to 2,4-dinitrophenylhydrozone (DNP-hydrozone). The samples were then blotted onto nitrocellulose. The membrane was then exposed to a DNP specific primary antibody. Following incubation with the primary antibody the membrane was incubated with an HRP-conjugated secondary antibody. The presence of the antibody complex was detected by chemiluminescence.

[00100] Cation-Exchange HPLC assay. cABCI oxidation products were analyzed using Dionex ProPac WCX-10 Cation Exchange column attached to Thermo Finnigan Chromatographic system consisting of Surveyor PDA detector, pump & autosampler. The enzyme was eluted with a NaCl gradient in 10 mM NaPhosphate buffer, pH 6.0. The detector's wavelength was set to 215 nm.

[00101] Size exclusion chromatography. Analytical size exclusion chromatography was performed through a KW-803 column (Showdex Inc.), which has a separation range of about 50 to 150,000, and a KW-804 column, which has a separation range of about 100,000-600,000, using HPLC (ESA Inc.) supplied with light-scattering (Wyatt Technology) and UV detector (Waters Co.). 100 mM NaPhosphate, pH 7.4 was used as a mobile phase.

[00102] Stress studies of cABCI. The lyophilized cABCI enzyme was reconstituted in a buffer of choice. The enzyme was allowed to reconstitute either on ice or at about 4°C for several hours and insoluble material, if any, was removed by centrifugation at 14,000g. Then 100µl aliquots were subjected to different stress conditions, including but not limited to, temperature, continuous vortexing, freeze-thawing, UV light, hydrogen peroxide presence. Samples subjected to UV light were kept on ice during exposure to minimize any

heating effect on the enzyme. Vortexing was done at about 4°C. Oxidation by hydrogen peroxide was tested by incubating samples with different hydrogen peroxide concentrations overnight at 4°C. Freeze-thawing cycles were executed on dry ice. Post-treatment samples were assayed for protein concentration by A280 readings, enzyme activity by spectrophotometry and further assessed by reducing and non-reducing SDS-PAGE, denatured IEF-PAGE, SEC and cation-exchange HPLC.

[00103] Formulation studies for cABCI. Since cABCI may be susceptible to heat inactivation, incubation at about 37°C was also used as a stressor in formulation studies. The reconstituted cABCI samples were incubated overnight or longer in a 37°C water bath with different additives and buffer components. After incubation, the samples were assayed for enzyme activity.

EXAMPLE 1

[00104] Recombinant chondroitinase ABCI was overexpressed in *E.coli* and purified according to the following steps:

- (i) enzyme extraction with Triton X-114/PBS and sonication from bacterial cell pellet;
- (ii) SP cation-exchange chromatography in sodium acetate buffer at pH 5.5;
- (iii) Sephacryl S200 gel filtration chromatography in sodium acetate buffer at pH 5.5;
- (iv) filtration through Q anion-exchange membrane for endotoxin and DNA removal; and
- (v) dialysis into a volatile buffer (ammonium bicarbonate at pH 8.0). Optionally this step may be followed by lyophilization, or by any other methods of concentrating and removal of buffer (for example, sterile filtration followed by suspending in an appropriate formulation).

[00105] A recombinant chondroitinase ABCI was overexpressed in *E. coli*. A large portion of the enzyme was released into solution with non-ionic detergent and sonication. An SDS-PAGE, visualized with silver stain, of the detergent-soluble extract and detergent-insoluble pellet fractions from ABCI-overexpressing bacterial cells revealed a single large band running between size markers of about 75 kDa and 100 kDa.

[00106] Cation-exchange chromatography was used as a capture step in further purifications. CEX SP chromatography at about pH 5.5 in acetate buffer was effective for capturing chondroitinase ABCI from the bacterial cell extract. It was found that the enzyme was bound quantitatively and eluted from the SP column with relatively high purity and yield when Triton X-114, final concentration in the range of about 0.2-1%, detergent was used in the extraction buffer. SDS-PAGE analysis of chondroitinase ABCI start, flow-through and elution fractions (25%B) from SP column for Triton X-114 extract revealed that the chondroitinase ABCI was eluted. A Triton X-100-containing extract appeared to alter the chondroitinase ABCI charge characteristics resulting in poor capture, poor elution yields and low step purity.

[00107] Removal of endotoxin was achieved by two methods, specifically partitioning into Triton X-114 and Q anion exchange membrane filtration.

[00108] SDS-PAGE analysis of chondroitinase ABCI fractions during endotoxin removal step using Triton X-114 partitioning method detected a single large band in the absence of any background bands.

[00109] Filtration through Q membranes was employed for the endotoxin removal step. This method was tested at about pH 5.5. 20 mM NaAcetate, pH 5.5, 100 mM NaCl was found to be an effective buffer for endotoxin binding to Q membranes (according to K.C. Hou and R. Zaniewski, Biotech. Appl. Biochem. 12, 315-324, 1990, these pH and salt conditions are expected to remove approximately 75% endotoxin) and for minimizing cABCI losses during this step. Greater than 95% of the cABCI was collected in a

flowthrough mode. This step was performed following gel filtration at the end of purification, as described below.

[00110] Gel filtration was used as a polishing step for chondroitinase ABCI. Examples of gel filtration that can be used include Sephacryl S200 and Sephacryl S300. Sephacryl S200 and Sephacryl S300 were tested for their efficacy in separating aggregates and low molecular weight contaminants. Two different elution buffers (Buffer 1: 20 mM Tris at pH 8.0, 200 mM NaCl, 0.5% betaine and Buffer 2: 20 mM sodium acetate at pH 5.5, 100 mM NaCl) for each gel were tested and found to work equally well. The chondroitinase ABCI was eluted with the expected retention times without substantial loss in sample.

[00111] Figure 2 depicts purification of chondroitinase ABCI by gel filtration. Figure 2A is a chromatography profile for Sephacryl S300 26/60 column. Figure 2B is an SDS-PAGE analysis of chondroitinase ABCI elution fraction from Sephacryl S300 26/60 column in 20 mM Tris, pH 8.0, 200 mM NaCl, 0.5% betaine. Figure 2C is an SDS-PAGE analysis of chondroitinase ABCI elution fraction from Sephacryl S200 26/60 column in 20 mM NaAcetate, pH 5.5, 100 mM NaCl.

[00112] For lyophilization, the purified enzyme was dialyzed into volatile buffer of 0.1M NH_4CO_3 , at about pH 8.0.

[00113] A gradient (4-20%) SDS-PAGE was performed, under reducing conditions, on the samples from the capture cation exchange column (start extract, flow-through, wash and SP elution pool) and gel filtration steps (S200 elution pool). The gel was stained with Coomassie blue. Gel analysis showed that the purification steps removed relatively all cell debris and contaminants, yielding a relatively pure enzyme sample.**EXAMPLE 2**

[00114] This example illustrates the enzyme activity of the purified recombinant chondroitinase ABCI of Example 1 as compared with the native enzyme. Chondroitinase ABCI enzymatic activity was assayed as described elsewhere herein. The

recombinant chondroitinase ABCI of Example 1 of the present invention has the same or higher specific activity as the native enzyme, and much higher activity as the recombinantly expressed chondroitinase ABCI as shown below in Table 4.

Table 4. Enzyme Activity

<u>Enzyme Source</u>	<u>Specific Activity, U/mg</u>
Native chondroitinase ABCI (Seikagaku)	120
Recombinant chondroitinase (Glyko)	24
Recombinant chondroitinase ABCI (Acorda)	160

[00115] SEC characterization was performed on both the recombinant chondroitinase ABCI of Example 1 and native chondroitinase ABCI as described above. The elution profiles of recombinant chondroitinase ABCI and the native chondroitinase ABCI were the same. The recombinant chondroitinase ABCI of the present invention had a retention time and molecular weight expected for a chondroitinase ABCI.

[00116] Determination of isoelectric point for the recombinant chondroitinase ABCI. IEF-PAGE, as described elsewhere herein, was used to determine the pI value for the recombinant chondroitinase ABCI of Example 1. The recombinant protein of Example 1 exhibited 3 isomeric forms with a pI of about 7.8-8.0 for the major isoform. This value was higher than expected for a native enzyme.

EXAMPLE 3

[00117] Chondroitinase ABCI, AC, and B were tested on a series of substrates and on rat spinal cord for specificity and activity using an improved anion exchange HPLC method. This method detects disaccharide CSPG cleavage products (Δ di-4DS and Δ di-6DS) with a quantification limit of 25 ng.

[00118] Measurements of the liberated disaccharide cleavage products revealed optimal enzyme concentrations, enzyme combinations, and substrate characteristics in rat spinal cord. The catalytic activity of chondroitinase ABCI and chondroitinase AC was synergistically enhanced with the addition of chondroitinase B. *Ex vivo* digestions of rat

spinal cord yield Δ di-4DS and Δ di-6DS in the ratio of about 95:5. Time course studies revealed that maximum product formation occurred within 6 hours even though the enzymes are active for much longer. Product inhibition was ruled out as a cause for this observation.

EXAMPLE 4

[00119] The following example illustrates the substrate specificity of the purified recombinant cABCI. cABCI (batch 7b) was reconstituted in 0.1 M sodium phosphate, and 50 mM NaCH₃COO at pH 7.4. Product formation rates were measured at different concentrations for chondroitins A, B, and C. The data were plotted and, where appropriate, fitted directly to the Michaelis-Menten equation for calculation of K_m and V_{max} values. The curves for chondroitin A, B and C exhibited saturation at high substrate concentrations that is typical for Michaelis-Menten kinetics. The following cABCI kinetic parameters were measured (Figures 1 and 3, Tables 3-5): K_m = 0.033 mg/ml and V_{max} = 283 U/mg for chondroitin A, K_m = 0.021mg/ml and V_{max} = 74 U/mg for chondroitin B, K_m = 0.025 mg/ml and V_{max} = 188 U/mg for chondroitin C. Considering impurity levels that are present in each substrate (approximately 70%, for chondroitin A, approximately 85% for chondroitin B and approximately 90% for chondroitin C), cABCI seems to have similar affinity for all chondroitins, but it digests them at different specific rates (chondroitin A > chondroitin C > chondroitin B). Table 5 provides the raw data for Chondroitin A, B and C concentration dependence curves.

Table 5. Activity

Substrate, mg/ml	Measured rates, nmole/min	Average activity, U/mg
Chondroitin A (70% purity) 0.032	5.03; 5.54; 6.8	127.9
0.048	6.85; 7.39; 7.15	157.6
0.08	9.50; 8.25; 10.38	207.2
0.12	11.34; 9.78; 11.41	239.6
0.16	11.65; 12.24; 9.19	243.6
0.24	8.97; 14.65; 10.46; 12.44	257.0
0.32	10.95; 10.45; 10.98	238.4
0.48	11.81; 11.48; 11.34	255.1
Chondroitin B (85% purity) 0.04	9.85; 10.49	47.1
0.06	12.97; 10.67	54.7
0.08	12.46; 12.88	58.7
0.16	14.47; 14.50	67.0
0.20	14.58; 12.72	63.2
0.40	15.20; 15.31	70.6
0.60	15.21; 13.98; 16.03	69.8
0.80	16.10; 14.97	71.9
Chondroitin C (90% purity)		
0.03	5.26; 3.91; 4.26	91.6
0.053	6.57; 6.41; 6.23	131.2
0.107	8.00; 8.55; 8.46	170.7
0.160	7.49; 7.98; 7.66	158.0
0.267	8.84; 8.49; 7.46	169.3
0.400	8.23; 9.23; 7.58	171.1
0.533	9.65; 8.33; 8.18	178.7

[00120] The average specific rates (U/mg) were plotted as a function of substrate concentration (mg/ml) and the data were directly fitted into the Michaelis-Menten equation for determination of cABCI kinetic parameters. Figure 3 shows the Michaelis-Menten curves for cABCI and its substrates: chondroitin A, B and C.

EXAMPLE 5

[00121] The inhibition of the purified chondroitinase ABCI enzyme in the presence of various divalent metal salts (1 mM) was measured. cABCI activity was assayed after metal additions were made. The inhibition capacity of the tested metals appears to be in the following order: Zn>> Ni>>Co>Ca>Mg. Notably, calcium and magnesium ions appear

to have some measurable inhibitory effects on cABCI. Table 6 provides the metal inhibition of the recombinant cABCI.

Table 6. Metal Inhibition

Metal salts, 1 mM	Measured rates, nmole/min	Retained activity, U/mg	% retained activity
None	12.6; 13.9; 14.7	127	100
CoCl ₂	6.5; 6.6	61	48
NiSO ₄	1.8; 1.9	17	14
ZnCl ₂	0.23; 0.29	2.4	2
CaCl ₂	7.6; 9.2	78	61
MgCl ₂	9.1; 9.2	85	67
FeCl ₂	interfered with an assay	ND	ND
Cu(CH ₃ COO) ₂	interfered with an assay	ND	ND

EXAMPLE 6

[00122] This example illustrates the effect storage buffer pH has on the stability of the purified recombinant cABCI. The lyophilized cABCI was reconstituted in 20 mM NaAcetate, pH 5.5, 100 mM NaCl buffer at 2.0 mg/ml concentration (using a BCA protein assay). Various pH conditions were achieved by diluting the reconstituted sample at about 1:2 ratios with 50 mM Bis-Tris propane buffers with various pH conditions. The cABCI concentration of the final samples was about 1mg/ml. The samples were stored at about 4°C and their activity was measured at 24 hrs, 48 hrs and 72 hrs. Table 7 provides the measured activity data for the recombinant cABCI samples stored under different pH conditions.

Table 7. Measured Activities

Storage pH	Starting rates, nmol/min	Rates after 24hrs at 4°C, nmole/min	Rates after 48hrs at 4°C, nmole/min	Rates after 72hrs at 4°C, nmole/min	Average rates, nmole/min
4	1.63; 2.47	5.47; 5.28	5.16; 5.00	4.84; 5.59	4.4
5	2.55; 3.13	5.92; 4.09	4.00; 3.70	4.89; 4.59	4.1
6	3.93; 4.04	4.46; 3.74	2.64; 2.78	4.90; 4.67	3.9
7	4.15; 5.55	4.41; 4.57	4.97; 5.09	4.91; 4.51	4.8
8	4.43; 3.49	3.62; 4.21	4.29; 4.04	4.24; 4.80	4.1
9	4.47; 3.65	4.53; 4.27	5.09; 4.49	5.05; 4.10	4.5

[00123] No significant differences were observed between the samples stored at different pH conditions. pH 7.4 is preferred in order to be within the physiological pH range.

EXAMPLE 7

[00124] The recombinant cABCI was subjected to different stress conditions. The activity and protein concentration data for the recombinant cABCI samples after various stress treatments are presented in Table 8.

Table 8. Activity Following Stress Conditions

Sample#	Treatment	Treatment points	Concentration, mg/ml	Measured rates, nmole/min	Activity, U/mg
Control	4°C		0.976	31.7; 30.0	126.4
1	Freezing	1 cycle	0.952	30.1; 29.2	124.6
2	Freezing	2 cycle	0.952	22.8; 27.8	108.6
3	Freezing	3 cycle	0.932	27.3; 25.8	114
4	H ₂ O ₂	0.5 mM	0.952	24.3; 22.7	98.8
5	H ₂ O ₂	5 mM	0.964	4.3; 6.1	21.3
6	H ₂ O ₂	20 mM	0.982	1.1; 1.4	5.15
7	Vortex	5 min	0.765	18.4; 17.9	94.8
8	Vortex	20 min	0.432	13.0; 12.3	117
9	Vortex	60 min	0.066	0.7; 0.8	46
10	UV	40 min	0.976	0.1; 0.3	0.8
11	UV	1hr	0.976	0.1; 0.1	0.36
12	UV	2hrs	0.976	0.1; 0.1	0.33
13	37°C	1hr	0.922	18.9; 16.0	75.5
14	37°C	4hrs	0.934	15.8; 17.0	70.1
15	37°C	20 hrs	0.801	14.6; 8.8	58.3

[00125] The stress-treated samples were also analyzed by SEC with a light-scattering detector. The SEC light-scattering profiles of the cABCI non-treated (control) and stress-treated samples are shown in Figures 4A-4E.

[00126] The recombinant cABCI, at about 1 mg/ml, did not appear to be affected by 3 cycles of freezing and thawing. The enzyme was precipitated and inactivated in a time-dependent manner when vortexing was used as the stressor. Hydrogen peroxide exposure resulted in loss of activity in a concentration-dependent manner with the noticeable

changes in its isoform profiles on IEF-PAGE. Exposure to UV light had a negative effect on the activity of cABCI. The enzyme did not appear to be stable at 37°C and its loss of activity appeared to be time dependent. Thermally inactivated samples had a decrease in protein concentration and showed changes in the isoform profile similar, but in lesser degree, to the ones observed for hydrogen peroxide treated samples.

[00127] A weak cation exchange (CEX) HPLC method was developed in order to quantify cABCI oxidation products. Therefore, another stress study was conducted in order to identify and correlate the presence of oxidation products with the loss of enzymatic activity. Some stress treatments were repeated under milder conditions than the ones used in the previous study (UV exposure). The reconstituted cABCI samples (0.6mg/ml) were exposed to 2 different sources of UV light (long-distance and short- distance exposure) for about 0.5, 1, 3 and 5 minutes and thermal inactivation (about 37°C) in 20 mM NaAcetate, pH 5.5, 100 mM NaCl buffer. The samples were assayed for activity and by IEF-PAGE, SDS-PAGE, oxyblot and CEX-HPLC. The results are described below.

[00128] Following oxidation treatment, an additional peak emerged on CEX-HPLC and is presumed to be the oxidized chondroitinase in Figure 5. This peak increased with an increase in the UV exposure time. The total areas under the curve in the RP-HPLC chromatograms remained nearly the same.

[00129] Figure 5 shows a weak CEX-HPLC analysis of the UV-treated cABCI samples. Shown are chromatograms of cABCI before (Figure 5A) and following 0.5 (Figure 5B), 3 (Figure 5C) and 5 minutes (Figure 5D) of close UV (hand-held light source) exposure.

[00130] Table 9 provides the cABCI activity data for non-treated (control) and stress-treated samples.

Table 9. Activity for Stress Treated and Control Samples.

Samples	Rates, nmole/min,	Activity, U/mg,
Control	49.3; 40.9; 40.9	146.7
UV light long-distance		
0.5min	53.5; 37.9; 46.3 46.304 45.87	154.0
1min	34.0; 44.6; 44.0	137.1
3min	37.62; 39.3; 32.4	122.2
5min	20.4; 29.2; 23.9	82.04
UV light short-distance		
0.5min	41.0; 36.0; 35.2	125.5
1min	16.3; 19.8; 19.2	61.9
3min	8.6; 9.3; 7.8	28.7
5min		
1 day at 37°C	13.3; 18.0; 14.1	50.9

[00131] Following exposure to UV and heat, cABCI samples were assayed for activity by the previously described spectrophotometric assay. There appears to be a correlation between the relative decrease in area of unoxidized cABCI peak with the relative decrease in activity (U/mg). Table 10, shown below, is the correlation of the decrease in the non-oxidized cABCI with the activity after exposure to short-distance and long-distance UV light.

Table 10. Activity After UV Exposure

Time, Mins	Peak Area	Control Peak Area	% of Initial Area	% of Initial Activity
0	19239	2739978	100.0	100.0
UV light short-distance				
0.5	93587	2429091	88.7	85.6
1	171634	1225441	44.7	42.1
3	18 0745	292674	10.7	19.7
5	181961	216126	7.9	6.4
UV light long distance				
0.5	31900	2770293	101.1	105.0
1.0	28732	2670994	97.5	93.6
3.0	68466	2410100	88.0	83.3
5.0	89149	1877997	68.5	55.8

[00132] Figures 6-8 show the correlation of the presence of the cABCI oxidized product with reduction in enzyme activity. Some correlation between the appearance of the oxidized product and the enzymatic activity was also observed for thermally inactivated samples. cABCI samples were exposed to heat and assayed for activity by spectrophotometry and for oxidized product by CEX-HPLC as described above.

[00133] Table 11 shows data of cABCI activity measurements after incubation at 37°C for 0 to 24 hours. One vial of cABCI Batch 7b was reconstituted in 50mM sodium acetate/ 100mM sodium chloride. Samples were incubated at 37°C for 1, 2, 4, 6 & 24 hrs. The concentration of the control sample (prior to any incubations) was determined. The sample's A₂₈₀ was 0.88, and the concentration was calculated to be equal to about 0.53mg/mL.

Table 11. Activity After Incubation

Samples	Measured rates, nmole/min	**Activity, U/mg
Control, 4°C*	34.4; 26.7	181.85
1 hr	26.6; 27.5	161.17
2 hr	22.0; 26.7; 21.3	138.87
4 hr	21.0; 21.5	126.56
6 hr	19.9; 11.6; 17.9; 9.6	87.79
24 hr	4.7; 4.7	27.91

[00134] Table 12. Correlation of cABCI activity data with HPLC profile of cABCI during thermal inactivation.

Table 12. Activity After Thermal Inactivation

Time	Peak Area	Control Peak Area	% of Initial Area	% of Initial Activity
Initial	49607	3342595	100	100.0
1 hr	60633	2926126	87.5	84.7
2 hr	77000	2728818	81.6	72.8
4 hr	88621	2068080	61.9	66.6
6 hr	134161	1375960	41.2	45.9
25 hr	311574	336266	10.1	14.7

[00135] Following activity determination and weak CEX-HPLC analysis, the thermally and UV- inactivated samples were also analyzed by SDS-PAGE, IEF-PAGE and Oxyblot.

[00136] The incubation at 37°C study was repeated for additional buffer conditions: 0.1M NaPhosphate, pH 7.4, 50 mM NaAcetate and 0.75M NaPhosphate, 50 mM NaAcetate, as described in Example 8.

EXAMPLE 8

[00137] In the following study, different buffers were used to determine enzyme stability. Recombinant cABCI was reconstituted in 50 mM NaAcetate, pH 6.5, 100 mM NaCl and diluted 1:3 with 0.2M solutions of the sodium acetate, sodium phosphate, Tris

and HEPES. Following an overnight incubation at 37°C in the different buffers the activity of cABCI was determined. The data are presented in Table 13.

Table 13. Activity based on Buffer System

Buffer system	Measured Rates, nmole/min	Activity, U/mg
Acetate, pH 6.5 after 4°C incubation	31.7; 30.0	126.4
Acetate, pH 6.5 after 37°C incubation	16.4; 20.8	82.4
Tris, pH 8.1 after 37°C incubation	15.5; 18.9	76.1
NaPhosphate, pH 7.4 after 37°C incubation	28.4; 28.0	124.8
HEPES, pH 6.8 after 37°C incubation	24.2; 26.9	113

[00138] This study revealed that the phosphate buffer provided the most protection for cABCI against thermal inactivation.

EXAMPLE 9

[00139] This example demonstrates the effect of various protein stabilizers (buffers) and excipients on their ability to stabilize the cABCI. The results of this evaluation of different protein stabilizers and buffer conditions for cABCI buffer formulation are listed in Table 14.

Table 14. Activity based on Protein Stabilizers and Buffers

Treatment	Starting Rates, nmole/min	Starting Activity, U/mg	Rates 72hrs, 37°C, nmole/min	Activity 72 hrs, 37°C, U/mg	% Activity Retained
Control: 100mM NaPhosphate pH 7.4, 50mM NaAcetate, 50mM NaCl	8.6; 9.8	91.6	2.6; 2.4	5.3	5.8
500mM NaCl	7.9; 10.0	89.5	14.2; 12.3	17.7	19.7
750mM NaPhosphate	11.3; 12.2	117.9	11.3; 11.8	106.0	90.0
0.1mM Hydroquinone	10.3; 10.2	102.9	0.03; 0.09	0	0
1% Mannitol	11.6; 9.2	104.5	4.6; 6.1	7.6	7.2
0.3M Sucrose	10.5; 8.9	87.3	13.0; 13.7	13.3	15.3
10% Glycerol	9.6; 10.2	98.8	3.6; 3.1	3.5	3.6
100mM Arginine	10.2; 11.6	109.2	0.06; 0.12	2.5	2.3
0.3M Trehalose	8.7; 8.7	87.1	0.8; 0.8	1.2	1.4
50mM NaPhosphate	3.0; 3.0	51.4	0.2; 0.3	3.3	6.4
50mM NaPhosphate 0.01% Polyethylimid	4.5; 4.3	68.3	2.2; 3.0	11.3	16.5

* shown are examples from two trials

[00140] Table 15 provides an evaluation of EDTA as additive to cABCI buffer formulation.

Table 15. Activity following EDTA Administration

Treatment	Starting rates, nmole/min	Starting Activity, U/mg	Rates 36hrs, 37°C, nmole/min	Activity 36 hrs, 37°C, U/mg	% Activity Retained
50mM NaPhosphate	5.9; 6.1	83.3	23.9; 26.0	53.1	63.7
50mM NaPhosphate 1 mM EDTA	6.0; 4.8	75.0	18.0; 21.1	65.2	86.9
750mM NaPhosphate	12.0; 10.8	158.2	5.9; 6.2	151.1	95.5
750mM NaPhosphate 1 mM EDTA	11.3; 8.2	135.8	5.9; 6.0	149.5	110.1

[00141] None of the excipients or buffers, except sodium phosphate at 750 mM, was effective against cABCI thermal inactivation. 500 mM NaCl also showed some improvement in cABCI thermal stability. The results suggested that ionic strength might play a role in protection of cABCI from thermal inactivation.

EXAMPLE 10

[00142] This example demonstrates the effects different salts and different salt concentrations have on the stability of cABCI using sodium chloride in the cABCI formulation buffer. Recombinant cABC was reconstituted in 50mM Na Phosphate pH 7.4 at approximately 2mg/ml. An initial activity reading was taken to determine the base level of activity. Chondroitinase (1mg/ml) was diluted into sodium chloride (NaCl) in 50mM Na Phosphate, pH 7.4 at concentrations ranging from 0 to 1M. The samples were allowed to incubate at 37°C for 48 hours. After two days the samples were assayed for activity. The data are presented below in Table 16-18 and Figures 10 and 11. Table 16 shows that thermal stability of cABCI is dependent on the on ionic strength of the NaCl in the presence of 50 mM Na Phosphate buffer.

Table 16. Thermal Stability and Ionic Strength

NaCl, mM	Measured Rates, nmole/min	Activity 48 hrs, 37°C, U/mg	Retained Activity, %
0	0.33; 0.33	3.3	5.1
10	0.11; 0.27	2.0	3.0
100	0.79; 0.92	8.5	13.2
250	0.83; 0.84	8.4	12.9
500	1.30; 1.76	15.3	23.7
1000	1.80; 1.67	17.4	26.9

[00143] Figure 10 is a graphic representation of the data shown in Table 16. Table 17 shows the effects of buffer concentration on the thermal stability of cABCI is dependent on the on the concentration of the buffer in the presence of 100 mM NaCl and 50 mM NaAcetate.

Table 17. Thermal Stability and Buffer

Phosphate buffer, mM	Rates, nmole/min	Activity 96 hrs, 37°C, U/mg	% Retained Activity
50	0.10; 0.07	0.83	0.8
100	4.4; 4.6	9.0	8.8
250	18.6; 20.8	43.9	43.0
500	16.4; 15.3	69	67.6
750	24.6; 20.8	103.3	101.4
1000	16.9; 19.9	92.2	90.5

[00144] Figure 12 is a graphic representation of the data presented in Table 17. The data showed that the increase in ionic strength appears to improve the cABCI thermal stability. 750 mM Na Phosphate also provides protection for cABCI.

[00145] The next set of experiments was performed in order to optimize the ionic strength conditions while maintaining the lowest possible salt concentration in the final formulation. The thermal stability of cABCI was determined in a solution of sodium phosphate and sodium sulfate. Sodium sulfate is known for its protein stabilizing effects. The concentrations of both salts were varied and the catalytic rates of cABCI were measured following incubations at 37°C for 19hrs, 48hrs, 120hrs, and 192hrs..

[00146] Table 18 depicts optimization of the sodium phosphate and the sodium sulfate concentrations for the cABCI buffer formulation. The cABCI was reconstituted in 0.1M Na₂HPO₄, 50 mM NaAcetate, pH 7.4. All samples had cABCI concentrations of 0.37mg/ml, and the same acetate concentration (50 mM), same pH (7.4) but different phosphate and sulfate concentrations. The samples were maintained in a 37°C water bath for the indicated times (19 hrs, 48 hrs, 120 hrs, and 192 hrs).

Table 18. Formulations of Sodium Phosphate and Sodium Sulfate

Sample	Rate after 19 hrs, nmole/min (Activity, U/mg)	Rates after 48 hrs, nmole/min (Activity, U/mg)	Rate after 120 hrs, nmole/min (Activity, U/mg)	Rate after 192 hrs, nmole/min (Activity, U/mg)
Control, 4°C 0.37mg/ ml	33.701 35.646 40.353 (197.66)	N/A	N/A	N/A
0.05 M Na ₂ HPO ₄				
#1	0.185 0.2766 (1.25)	N/A	N/A	N/A
#2 0.15M Na ₂ SO ₄	0.984 0.777 (4.8)	N/A	N/A	N/A
#3 0.22M Na ₂ SO ₄	1.3588 1.3924 (7.44)	N/A	N/A	N/A
#4 0.3M Na ₂ SO ₄	1.9721 1.8339 (10.29)	N/A	N/A	N/A
0.1M Na ₂ HPO ₄				
#5	1.1044 0.9543 (5.56)	N/A	N/A	N/A
#6 0.15M Na ₂ SO ₄	1.6853 1.4162 (8.38)	N/A	N/A	N/A
#7 0.22M Na ₂ SO ₄	2.7917 2.5572 (14.46)	N/A	N/A	N/A
#8 0.3M Na ₂ SO ₄	3.3989 3.8001 (19.46)	N/A	N/A	N/A
0.1M Na ₂ HPO ₄		N/A	N/A	N/A
#9	3.9975 3.2614 (19.62)	N/A	N/A	N/A
#10 0.15M Na ₂ SO ₄	7.7154 5.48 (35.66)	N/A	N/A	N/A
#11 0.22M Na ₂ SO ₄	5.1651 4.9371 (27.3)	N/A	N/A	N/A

Sample	Rate after 19 hrs, nmole/min (Activity, U/mg)	Rates after 48 hrs, nmole/min (Activity, U/mg)	Rate after 120 hrs, nmole/min (Activity, U/mg)	Rate after 192 hrs, nmole/min (Activity, U/mg)
#12 0.3M Na ₂ SO ₄	8.4036 8.6568 (46.1)	2.64 3.24 (15.89)	0.06 0.23 0.1 0.02 (0.27) precip	N/A
0.4M Na ₂ HPO ₄				
#13	11.995 14.978 (72.9)	8.08 9.74 9.97 (50.07)	1.15 1.05 1.41 (0.45)precip	N/A
#14 0.15M Na ₂ SO ₄	20.921 17.78 (104.6)	17.2 15.5 (88.38)	5.29 5.25 (14.2)precip	N/A
#15 0.22M Na ₂ SO ₄	20.066 22.769 (115.77)	14.5 19.0 15.26 11.72 (81.7)	7.48 9.75 (23.3)precip	N/A
#16 0.3M Na ₂ SO ₄	18.07 16.462 (93.32)	16.4 18.1 12.8 19.0 (90.0)	13.4 12.4 8.2 (30.5)precip	N/A
0.75M Na ₂ HPO ₄				
#17 cABCI	30.481 27.388 28.128 (154.95)	22.3 28.7 30.9 26.1 (145.9)	20.3 7.0 9.1 13.8 (90.45)precip	13.234 13.749 (73)

[00147] The data for 19 hrs samples are summarized in Table 19 as percent activity remaining.

Table 19. Percent Activity After 19 Hours

Na ₂ HPO ₄ Na ₂ SO ₄	0.05M	0.1M	0.2M	0.4M	0.75M
0	0.6	2.8	9.9	37.0	78.7
0.15M	2.4	4.3	18.1	53.1	NA
0.22M	3.8	7.4	13.9	58.8	NA
0.3M	5.2	9.9	23.4	47.4	NA

[00148] Although sodium sulfate appears to improve the cABCI stability, the protection from phosphate appears to be more pronounced. 0.75M sodium phosphate buffer was selected for cABCI formulations for use at 37°C. This buffer was not selected for formulations that may be stored at lower temperatures due to its propensity to precipitate.

EXAMPLE 11

[00149] This example illustrates the effect of enzyme concentration on its thermal stability. Samples were suspended in 0.75 M phosphate pH 7.4, 50 mM Na Acetate. To determine the working range of cABCI concentrations, cABCI thermal stability at different enzyme concentrations was measured. It was observed that cABCI at low concentrations were as stable as cABCI at high concentrations (see Table 20). Also it was found that cABCI at high concentrations tends to precipitate after prolong exposure to 37°C. To avoid this problem, for example, cABCI concentrations may be kept below ~0.4mg/ml as shown in Table 20.

Table 20. 37°C stability of cABCI samples at different enzyme concentrations in 0.75M NaPhosphate buffer.

cABCI, mg/ml	Starting Rates, nmole/min (Activity, U/mg)	19 hrs at 37°C Rates, nmole/min (Activity, U/mg)	48 hrs at 37°C Rates, nmole/min (Activity, U/mg)	120 hrs at 37°C Rates, nmole/min (Activity, U/mg)	192 hrs at 37°C Rates, nmole/min (Activity, U/mg)
0.75	NA	26.3; 16.1; 21.9 (115.9)	21.8; 19.6; 27.1 (101.5)	precipitate	2.7; 2.5 (14)
0.35	33.7; 35.6; 40.3 (197.66)	30.5; 27.3; 28.1 (155.0)	22.3; 28.7; 30.9; (145.9)	precipitate	13.2; 13.7 (73)
0.18	NA	24.3; 24.3; 26.0 (134.36)	29.1; 26.6; 21.1 (138.4)	14.6; 20.6; 24.4 (107.4)	14.7; 15.3 (81)
0.09	NA	20.7; 21.9; 22.2 (116.79)	23.7; 21.4; 20.3 (117.8)	20.4; 18.0 (103.8)	11.0; 12.9 (65)

[00150] SDS-PAGE and Western blot analysis of the recombinant cABCI revealed a partial fragmentation of the cABCI after prolonged storage at 4°C. The

observation that degradation products are the same for both recombinant and native proteins suggests that the observed fragmentation may be due to an intrinsic property of cABCI.

[00151] The amino terminal end of the degradation products was sequenced. These results revealed that most of the degradation products were a mixture of protein fragments. Band #1: ATSNPAF (SEQ ID NO: 3); Band #2: ATSNPAF major (SEQ ID NO:4); NLNTSGD minor (SEQ ID NO:5); Band #3: ASNPADF (SEQ ID NO:6) plus a mixture of sequences; Band #4: $X_1X_2NX_3V-X_4-X_5$ (SEQ ID NO:7) mixture wherein X_1 can be A or N; X_2 can be T or P; X_3 can be T or E; X_4 can be A or G; and X_5 can be F or E; Band #5: $X_1X_2NX_3X_4X_5$ (SEQ ID NO:8) mixture wherein X_1 can be A or N; X_2 can be T or Y; X_3 can be T or P; X_4 can be A or E; X_5 can be A or G; Band #6: MQVNERD major (SEQ ID NO:9); GPRGAGT minor (SEQ ID NO:10); Band #7: no sequence was identified; Band #8: ATSNPAF (SEQ ID NO:11).

EXAMPLE 12

[00152] This example shows the results of a purification method for the purification of chondroitinase AC.

[00153] The cells expressing chondroitinase AC were extracted using a square tip sonicator at a maximum speed of about 9. Sonication was performed for about 30 seconds. This was immediately followed by about 10 seconds with no sonication. These on/off steps were performed for a total of about 10 cycles. Each pellet was sonicated separately and then pooled. Extractions were rocked overnight at 4°C.

[00154] Following cell extraction of the chondroitinase AC the sonicated sample was analyzed on SDS-PAGE to analyze the solubility of the chondroitinase AC protein. The enzyme was detected primarily in the supernatant, implying that the protein is soluble. Purification was continued using a cation-exchange column to capture the enzyme.

[00155] All of the cell extract was loaded onto a 20ml SP column. The extract was loaded onto the column at about 0.5 ml/min. The SP column was attached to the AKTA

Explorer to view wash and elution peaks. The column was washed and the chondroitinase AC was eluted. The column fractions were then analyzed on SDS-PAGE to check purity and gel analysis revealed that the chondroitinase AC eluted from about 245 mM NaCl to about 370 mM NaCl. The SDS-PAGE analysis revealed that the eluted fractions contained relatively pure chondroitinase AC and they were pooled resulting in a total volume of 190 ml. The 190 ml of pooled fractions were concentrated using a 10,000 MWCO membrane (Millipore) down to a total volume of 105 ml with an absorbance (A₂₈₀) of 1.47. The concentrated sample was then further purified using a gel filtration column.

[00156] Samples from the cation exchange step were loaded onto a S200 gel filtration column. . Samples were eluted using 20 mM sodium acetate, 100 mM NaCl pH 5.5. The first purification run through the gel filtration column was analyzed on SDS-PAGE to check purity. The fractions that revealed a relatively pure chondroitinase AC were pooled. Following the fifth purification run through the column the fraction samples were once again checked on SDS-PAGE for purity. The fractions revealing a relatively pure chondroitinase AC from all seven runs were pooled for a total volume of 250 mls with an absorption(A₂₈₀) of 0.431. The 250 mls were concentrated down to a total volume of 83 mls with absorption (A₂₈₀) of 1.40.

[00157] Removal of endotoxin from the 'chondroitinase AC sample was achieved by further purification of the sample isolated from the gel filtration step. Samples were spun through a Q anion exchange membrane (as described elsewhere herein) and chondroitinase AC was collected in a flowthrough mode. This method was tested at about pH 5.5. 20 mM sodium acetate, pH 5.5, 100 mM NaCl was found to be an effective buffer for endotoxin binding to Q membranes, these pH and salt conditions are expected to remove approximately 75% endotoxin. The resulting absorption(A₂₈₀) was 1.37. The final product was analyzed on SDS-PAGE for purity. The results revealed a pure chondroitinase AC with a molecular weight of about 50 to 75 kDa.

[00158] The purified chondroitinase AC was dialyzed into a volatile buffer of 0.1 M ammonium bicarbonate pH 8.0 overnight and aliquoted into small samples (about 1.0 ml), lyophilized and stored at -80°C. Figure 12 shows the SDS-PAGE of the final purified chondroitinase AC.

[00159] Although the present invention has been described in considerable detail with reference to certain preferred embodiments thereof, other versions are possible. Therefore the spirit and scope of the appended claims should not be limited to the description and the preferred versions contained within this specification.

What is claimed is:

1. A formulation comprising chondroitinase and phosphate buffer, wherein said formulation is stable for at least about 24 hours.
2. The formulation of claim 1, wherein said chondroitinase is purified.
3. The formulation of claim 1, wherein said chondroitinase retains at least about 50% of activity at about 24 hours.
4. The formulation of claim 1 wherein said chondroitinase is selected from the group consisting of chondroitinase, ABCI, chondroitinase ABCII, chondroitinase AC, chondroitinase B or mammalian enzymes with chondroitinase-like activity such as Hya11, Hya12, Hya13, Hya14 and PH20.
5. The formulation of claim 1, wherein said chondroitinase is chondroitinase ABCI.
6. The formulation of claim 1, wherein said chondroitinase is chondroitinase AC.
7. The formulation of claim 1, wherein said phosphate buffer is a sodium phosphate buffer.
8. The formulation of claim 1, wherein said sodium phosphate buffer is at a concentration of about 100 mM.
9. The formulation of claim 1 further comprising sodium acetate.
10. The formulation of claim 1, wherein said formulation is at a pH of about 7.4.
11. The formulation of claim 1, wherein said chondroitinase ABCI is an amino acid sequence of SEQ ID NO: 2.
12. A formulation consisting essentially of chondroitinase and a buffer.
13. A method of purifying chondroitinase comprising:
 - extracting said chondroitinase from said cells;
 - separating said chondroitinase from an extract;
 - removing contaminants and impurities; and
 - removing endotoxin.

14. The method of claim 13 wherein said chondroitinase comprises a recombinant chondroitinase.
15. The method of claim 14, wherein said recombinant chondroitinase is an amino acid sequence of SEQ ID NO: 2.
16. The method of claim 13, wherein said extracting includes suspending cells in a buffer solution containing a surface active agent.
17. The method of claim 16, wherein said surface active agent is Triton X.
18. The method of claim 13, wherein said extracting includes sonicating said cells;
19. The method of claim 13, wherein said separating said chondroitinase includes using a cation exchange column.
20. The method of claim 13, wherein said removing contaminants and impurities includes using a gel filtration column.
21. The method of claim 13, wherein said removing endotoxin includes using an anion exchange column.
22. The method of claim 13 further comprising dialysis.
23. The method of claim 22, wherein said dialysis includes using a volatile buffer.
24. The method of claim 13 further comprising drying.
25. The method of claim 24, wherein said drying is lyophilization.
26. The method of claim 13, wherein said chondroitinase is selected from the group consisting of chondroitinase, ABCI, chondroitinase ABCII, chondroitinase AC, chondroitinase B or mammalian enzymes with chondroitinase-like activity such as Hya11, Hya12, Hya13, Hya14 and PH20.
27. The method of claim 13, wherein said chondroitinase is chondroitinase ABCI.
28. The method of claim 13, wherein said chondroitinase is chondroitinase AC.
29. A chondroitinase purified according to claim 13.

30. A method of purifying chondroitinase comprising the steps of:
- extracting said chondroitinase from a cell;
 - cation-exchange chromatography;
 - gel filtration chromatography;
 - anion-exchange filtration; and
 - dialysis.

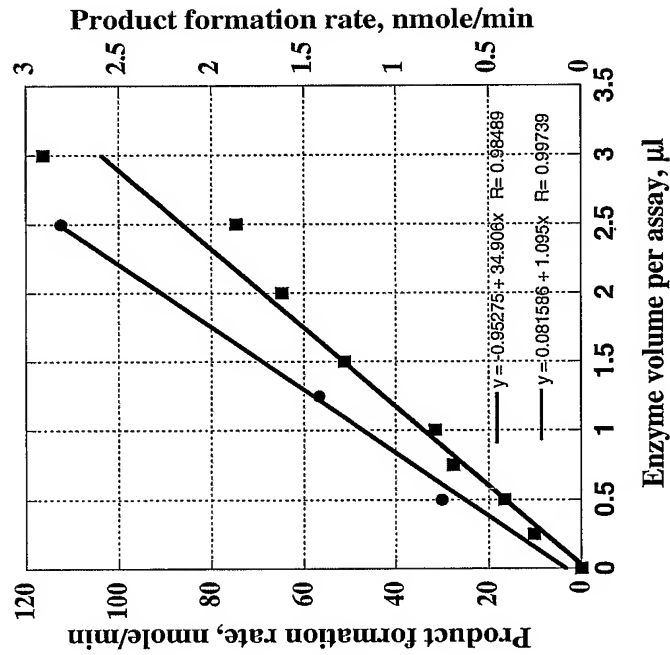


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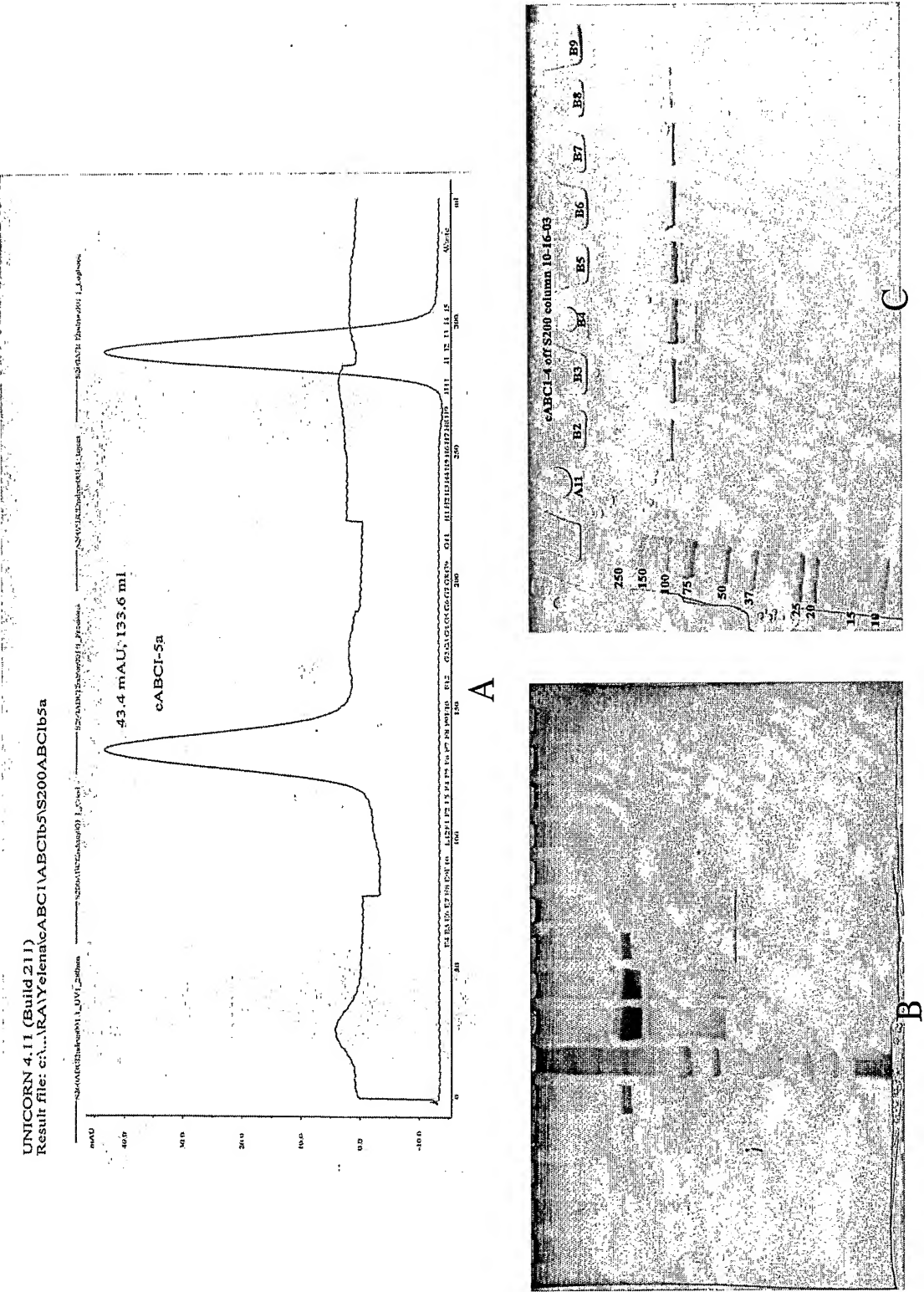


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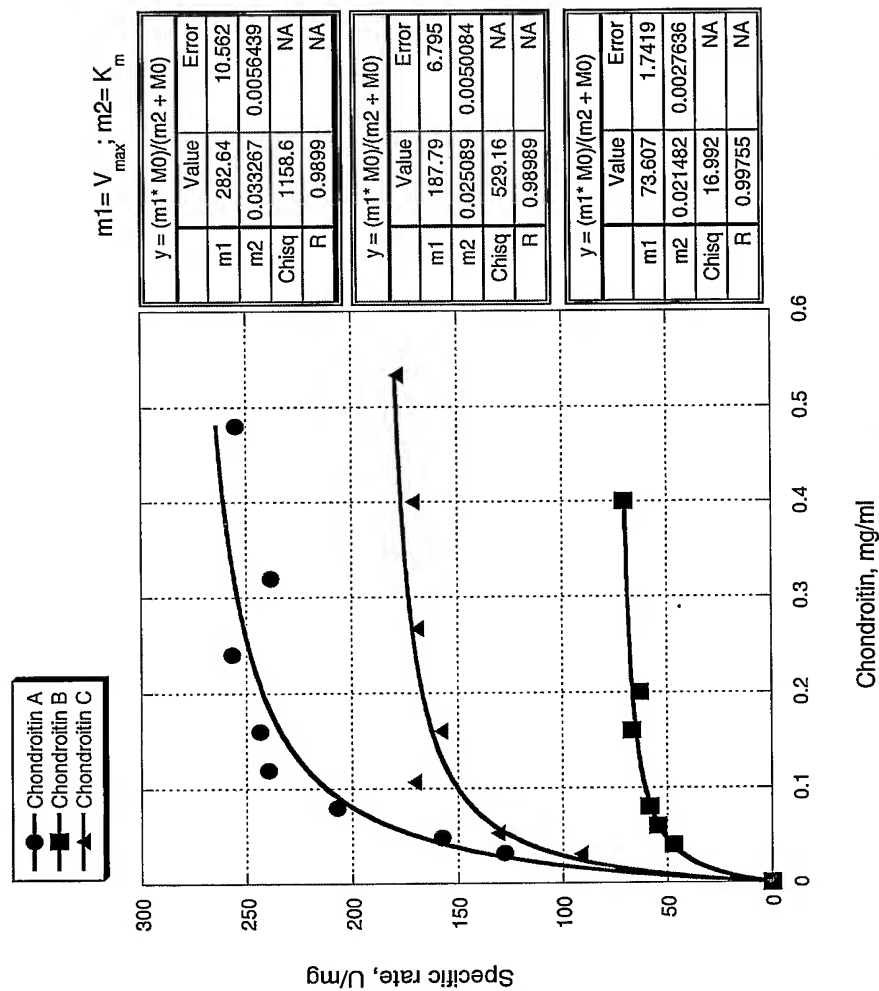


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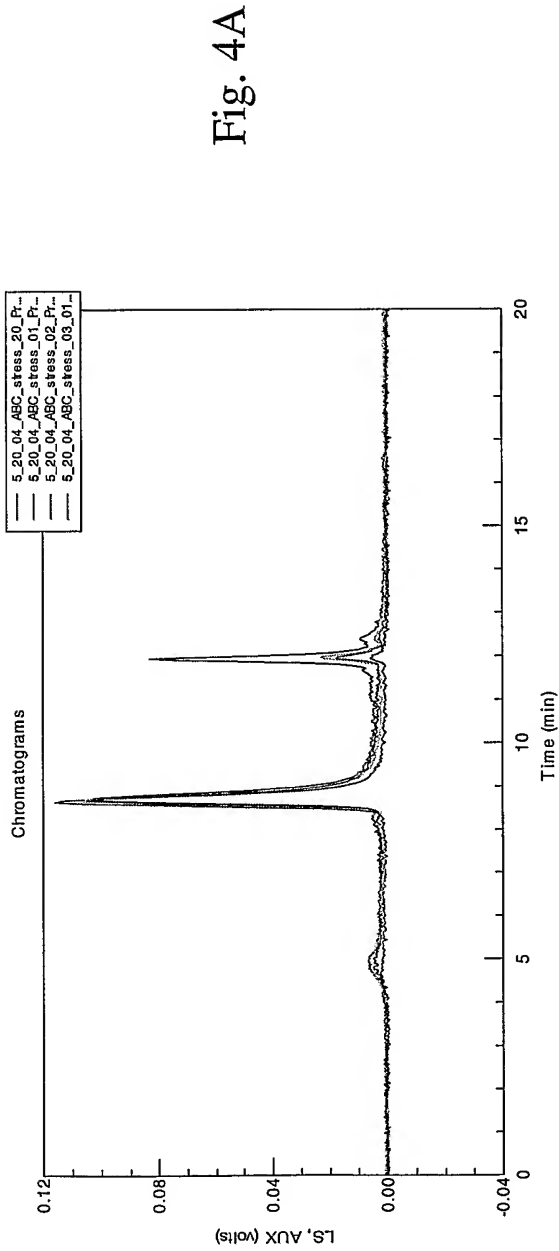


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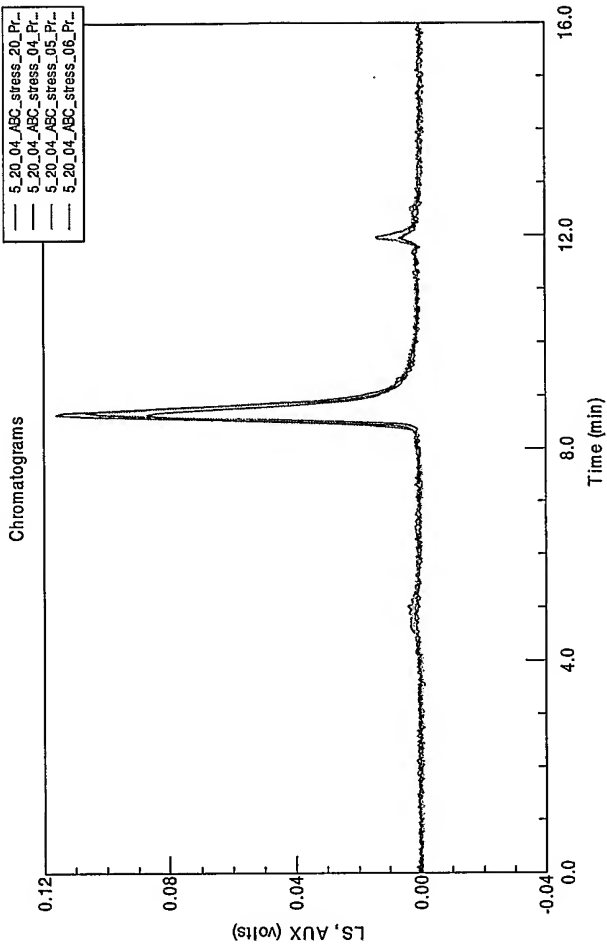


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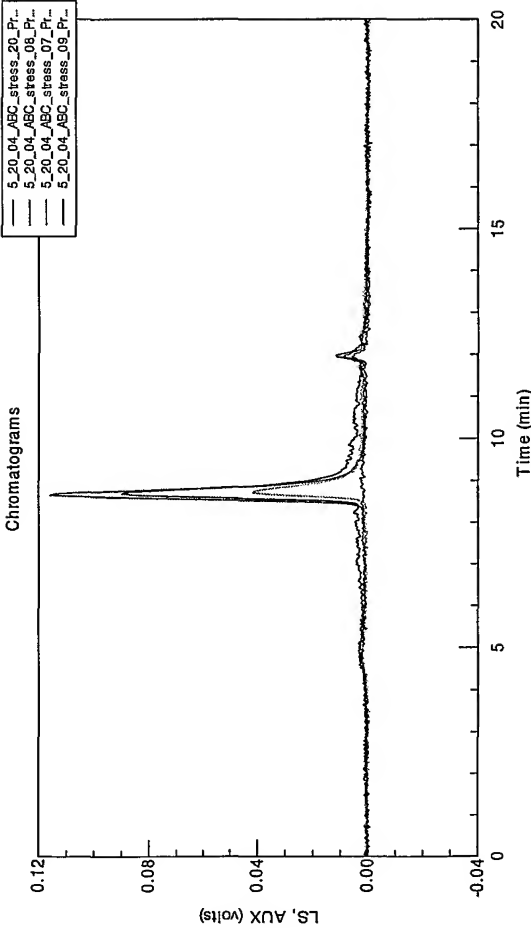


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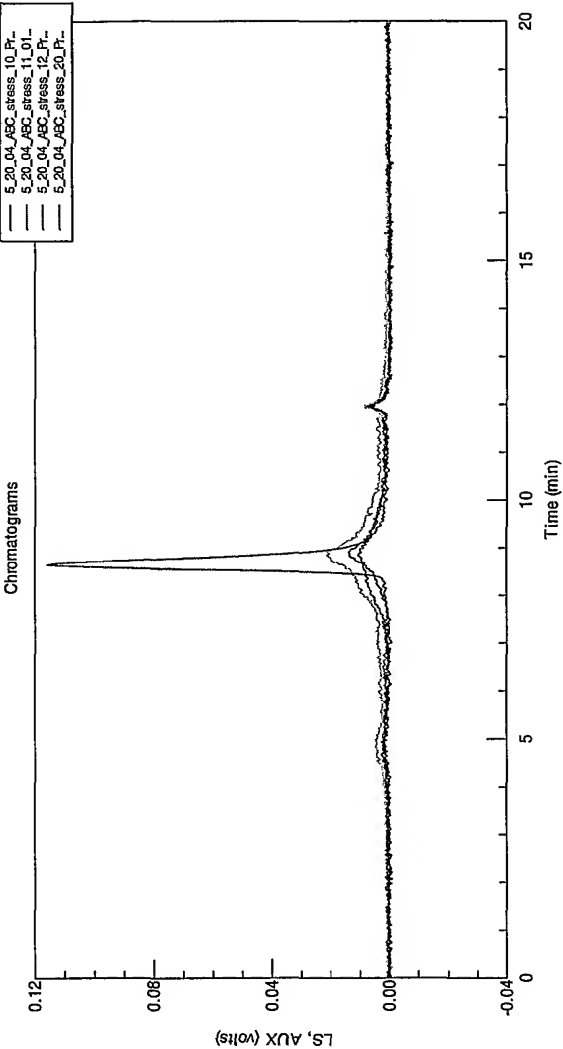


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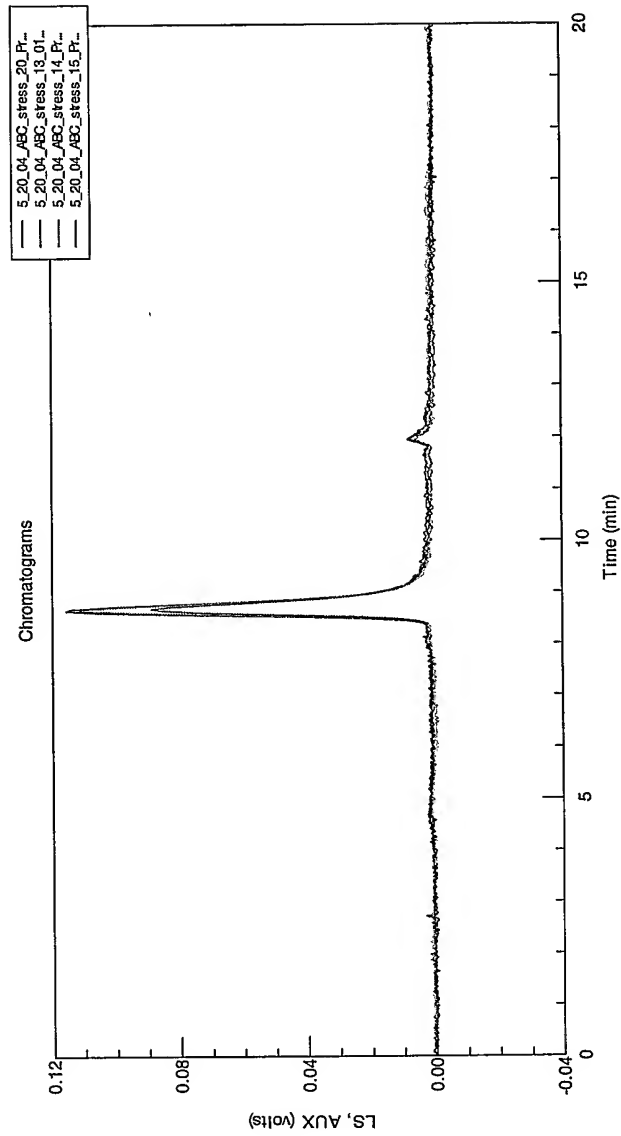


Fig. 4E

Fig. 5A

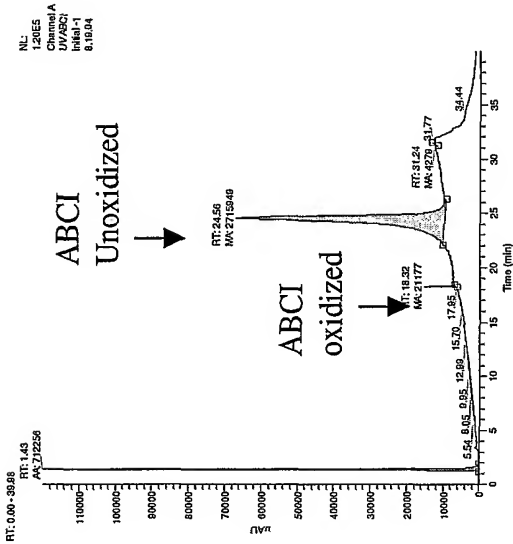


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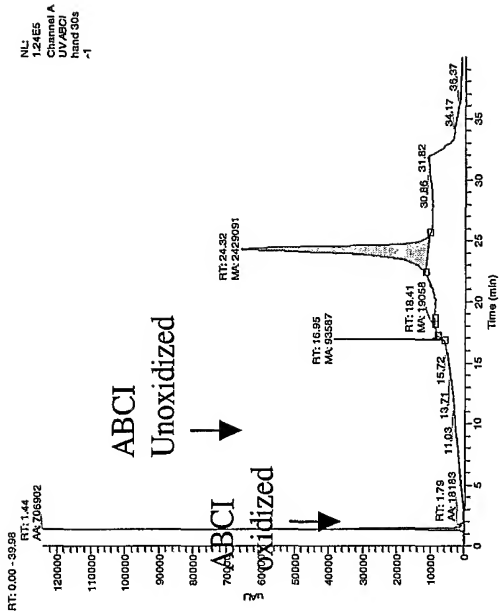


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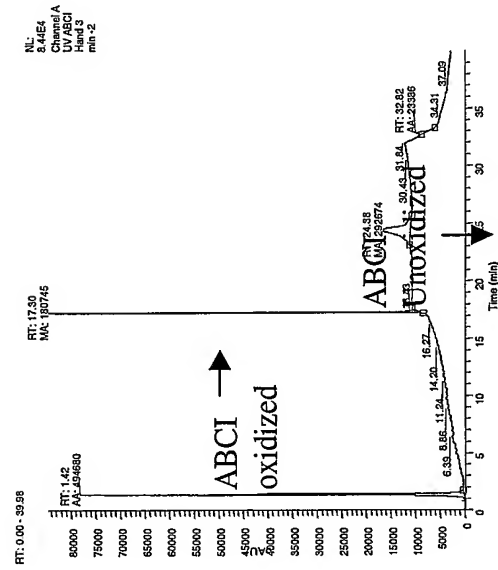


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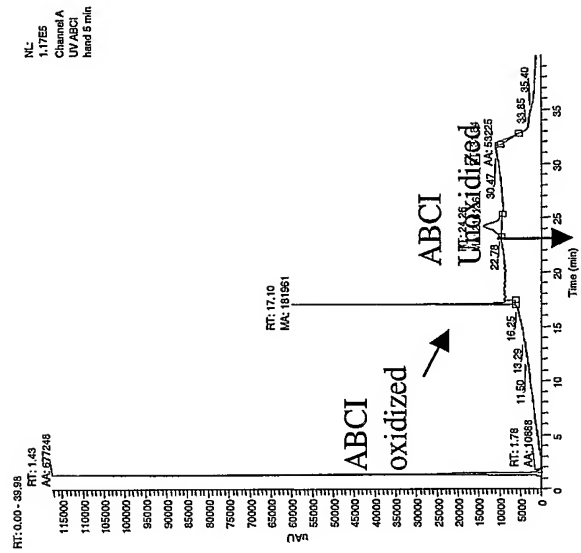


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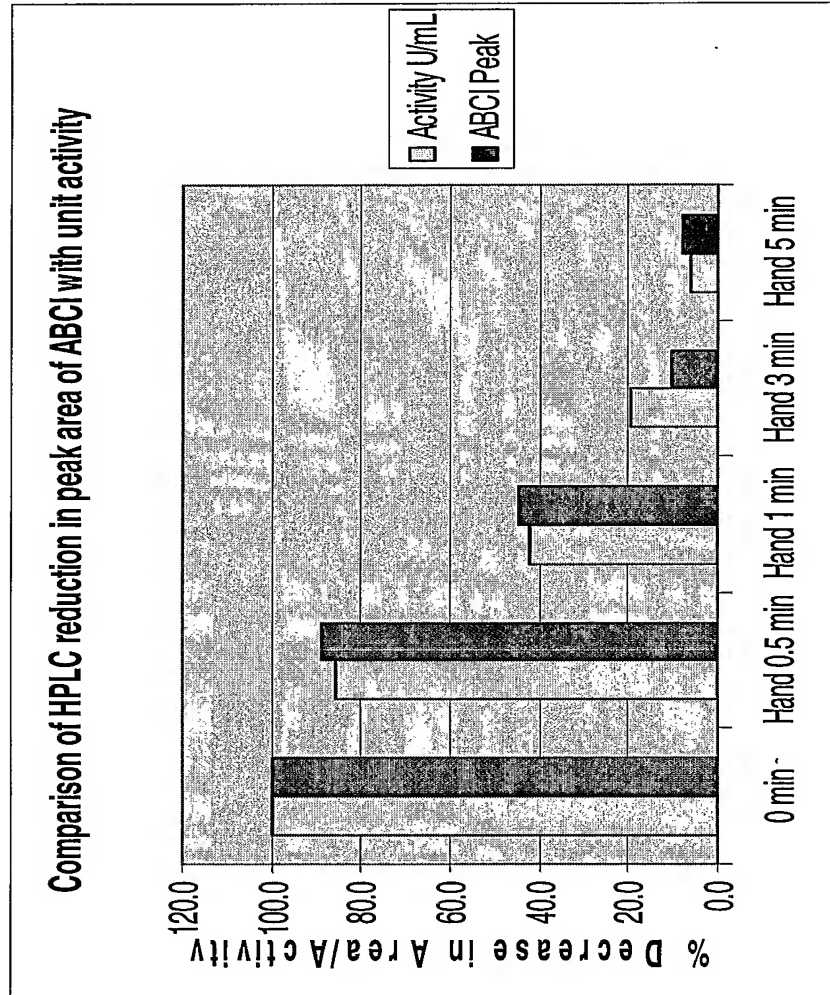


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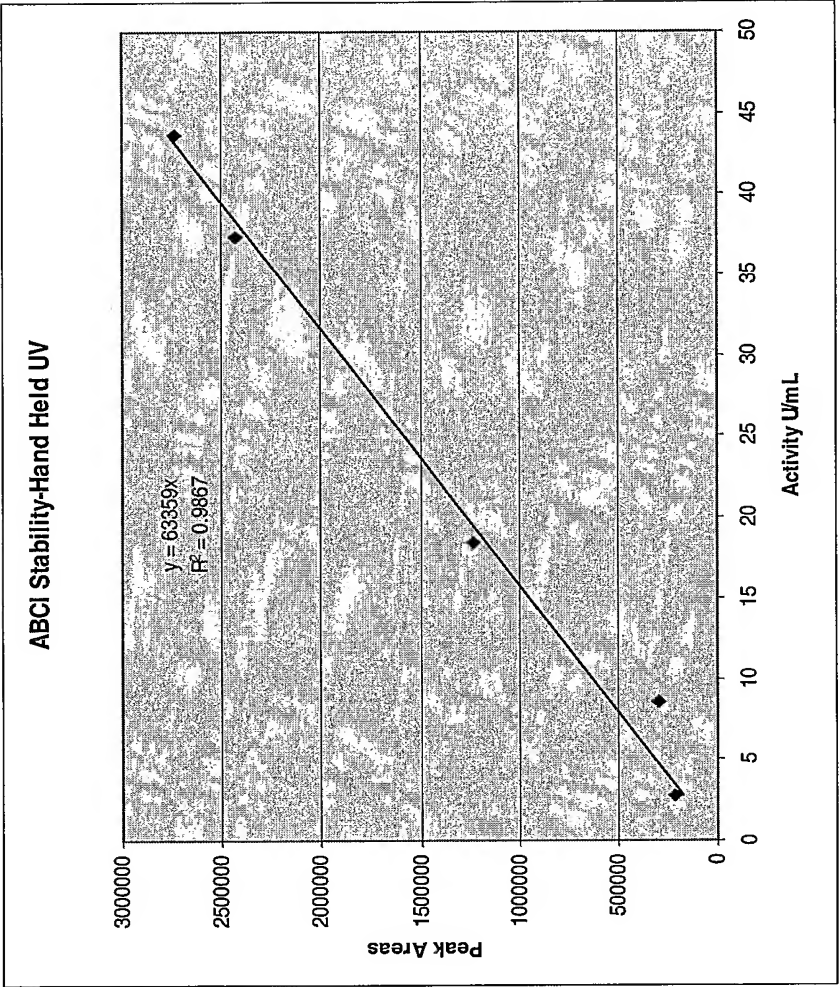
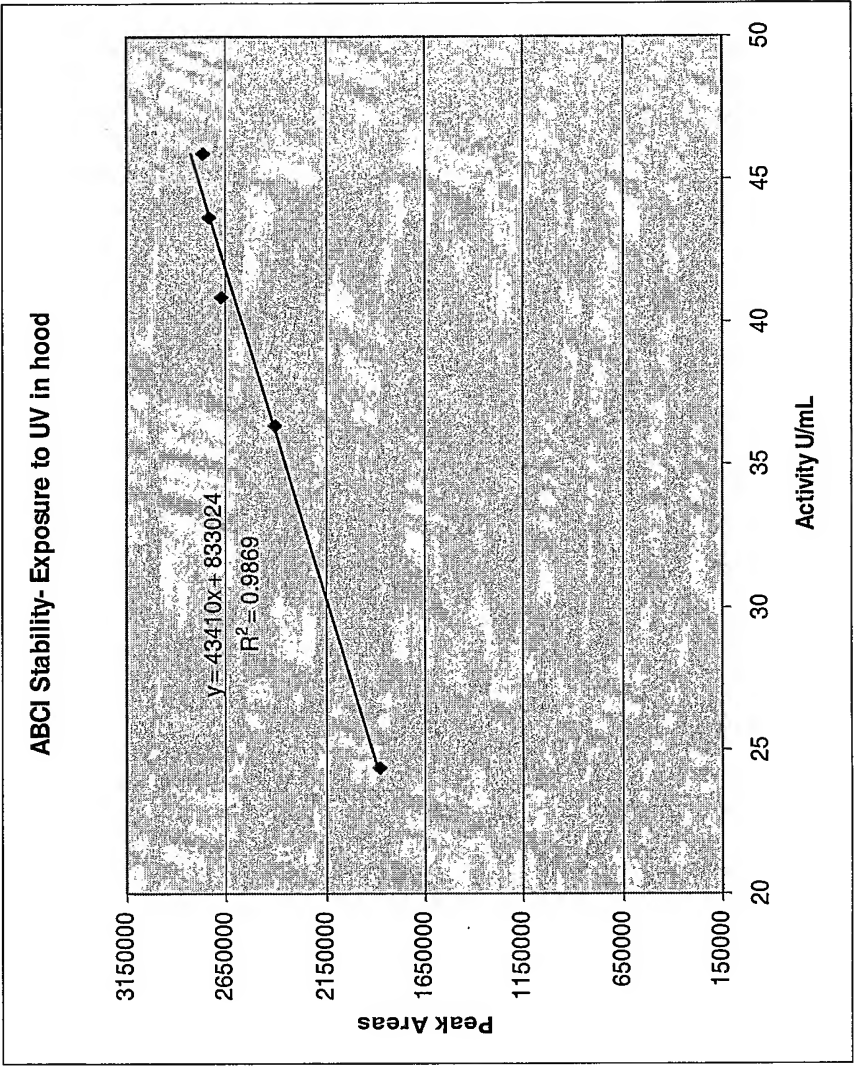


Fig. 8



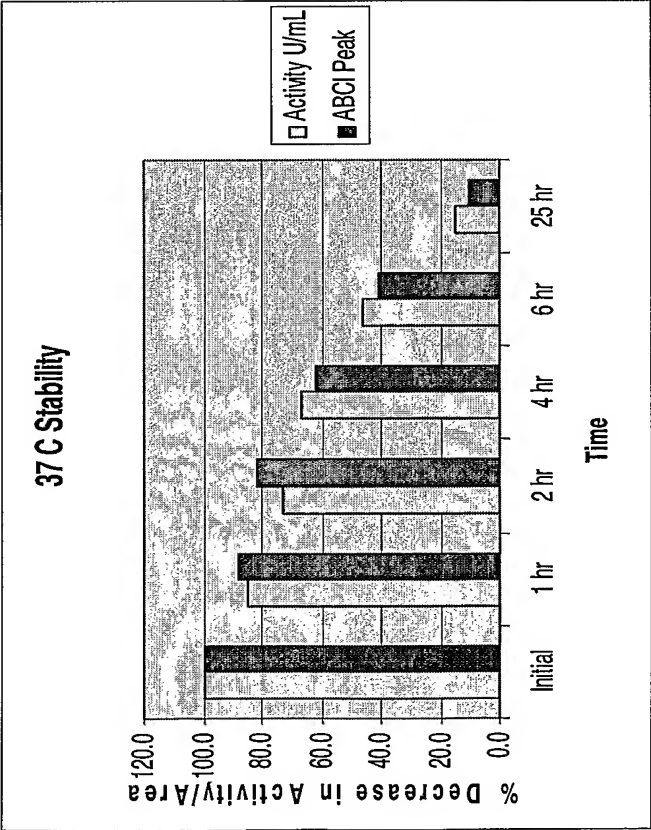


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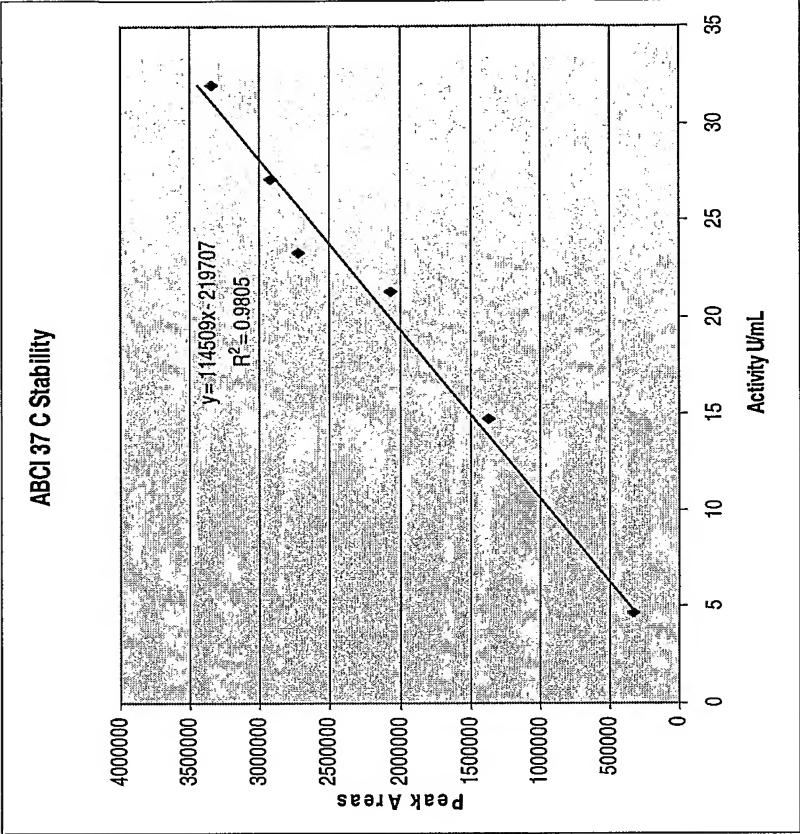


Fig. 9B

Fig. 10

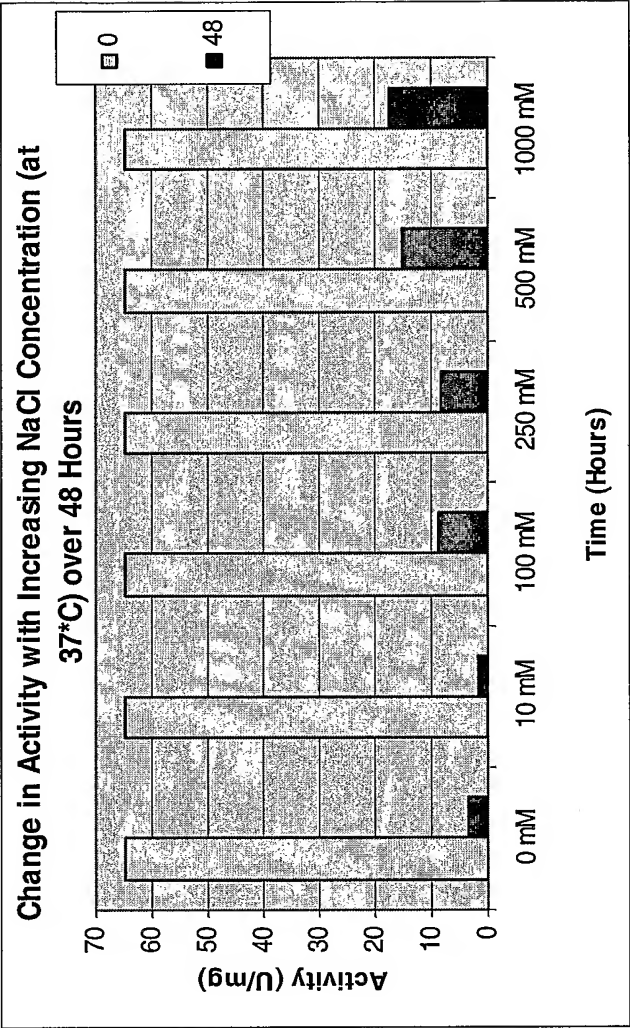
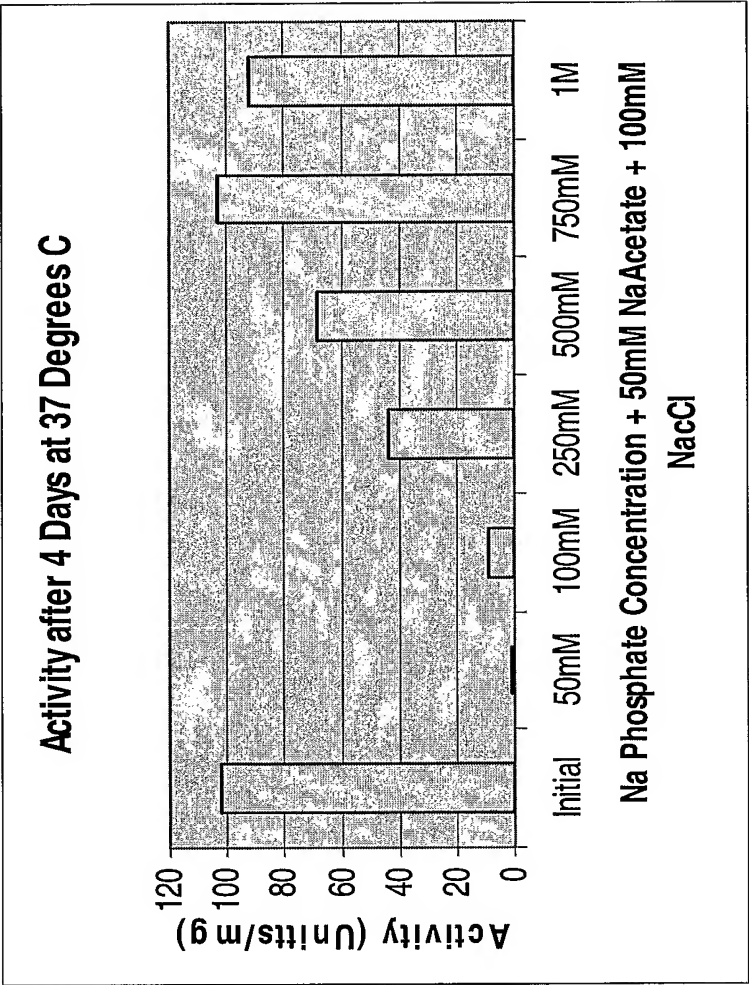


Fig. 11



Purity yield: 100%

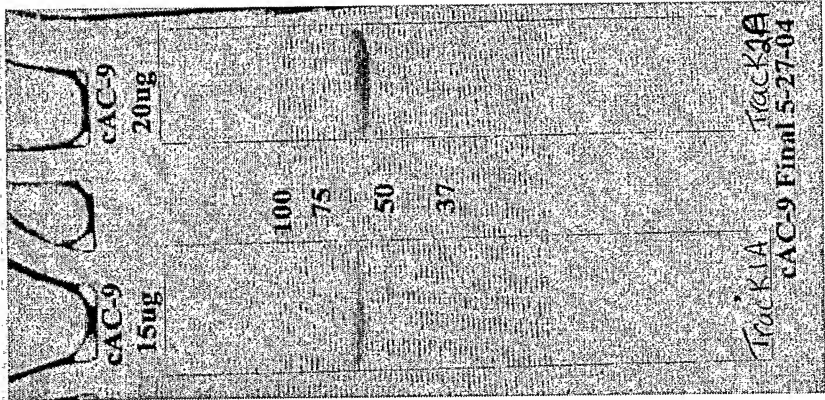


Fig. 12

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<223> Xaa can be T or Y
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<222> (4)..(4)
<223> Xaa can be T or P
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Ala Thr Ser Asn Pro Ala Phe
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